

Molecular mechanisms of apoptosis induced by dexamethasone in chronic lymphocytic leukemia

Maria João Gomes Monteiro Lopes Baptista

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**MOLECULAR MECHANISMS OF APOPTOSIS
INDUCED BY DEXAMETHASONE
IN CHRONIC LYMPHOCYTIC LEUKEMIA**

DOCTORAL THESIS

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PhD degree by the University of Barcelona

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ABBREVIATIONS

Abbreviations

ABBREVIATIONS

4EBP1 HGNC gene symbol for eukaryotic translation initiation factor 4E binding protein 1

ADAM29 HGNC gene symbol for ADAM metallopeptidase domain 29

ALL Acute Lymphoblastic Leukemia

AKT alias PKB; HGNC gene symbol: AKT1

ANXA1 HGNC gene symbol for Annexin A1

AP1 Activator Protein 1

APAF1 HGNC gene symbol for apoptotic peptidase activating factor 1

APC Allophycocyanin

ATM HGNC gene symbol for ataxia telangiectasia mutated

AU Arbitrary Units

BAD BCL2-associated agonist of cell death

BAG1 HGNC gene symbol for BCL2-associated athanogene

BAK BCL2-Antagonist/Killer 1; HGNC gene symbol: BAK1

BAX HGNC gene symbol for BCL2-associated X protein

BAFF B-cell Activating Factor; HGNC gene symbol: TNFSF13B

BCL2 HGNC gene symbol for B-cell CLL/lymphoma 2

BCL2A1 HGNC gene symbol for BCL2- related protein A1

BCLXL B Cell Lymphoma-extra large; HGNC gene symbol: BCL2L1

BCR B Cell Receptor

BID HGNC gene symbol for BH3 interacting domain death agonist

BIM BCL2-like 11 (apoptosis facilitator); HGNC gene symbol: BCL2L11

BM Bone Marrow

BMF HGNC gene symbol for Bcl2 modifying factor

CBG Cortico steroid Binding Globulin; HGNC gene symbol: SERPINA6

CCND1 HGNC gene symbol for cyclin D1

Abbreviations

cDNA Complementary DNA

CDR Complementarity Determining Region

ciAP1 Baculoviral IAP repeat containing 2; HGNC gene symbol: BIRC2

ciAP2 Baculoviral IAP repeat containing 3; HGNC gene symbol: BIRC3

CLL Chronic Lymphocytic Leukemia

CLLU1 HGNC gene symbol for chronic lymphocytic leukemia up-regulated 1

COX2 Cyclooxygenase 2; HGNC gene symbol: PTGS2

CREB cAMP Responsive Element Binding proteins family of transcription factors

CTLA4 HGNC gene symbol for cytotoxic T-lymphocyte-associated protein 4

CXCR4 HGNC gene symbol for Chemokine (C-X-C motif) receptor 4

DDIT4 HGNC gene symbol for DNA-damage-inducible transcript 4

DIABLO HGNC gene symbol for Diablo, IAP-binding mitochondrial protein; alias:
SMAC

DXM Dexamethasone

eNOS Endotelial Nitro Oxide Synthase; HGNC gene symbol: NOS3

EGF HGNC gene symbol for epidermal growth factor

ERK Extracellular-signal-Regulated Kinases; HGNC nomenclature: MAPK mitogen-
activated protein kinases

FAS HGNC gene symbol for Fas (TNF receptor superfamily, member 6)

FBS Fetal Bovine Serum

FBXO32 HGNC gene symbol for F-box protein 32

FC Fold Change

FCR Fludarabine, Cyclophosphamide and Rituximab

FCRL2 HGNC gene symbol for Fc receptor-like molecule 2

FDR False Discovery Rate

FISH Fluorescent *In Situ* Hybridization

FITC Fluorescein Isothiocyanate

FKBP4 HGNC gene symbol for FK506 Binding Protein 4, 59kDa

FKBP5 HGNC gene symbol for FK506 Binding Protein 5

FOXO1 HGNC gene symbol for forkhead box O1

FOXO3 HGNC gene symbol for forkhead box O3

FOXP3 HGNC gene symbol for forkhead box P3

FR Framework Regions

FSC Forward Scatter

FYN HGNC gene symbol for FYN oncogene related to SRC, FGR, YES

G6PC Glucose-6-phosphatase

GAPDH HGNC gene symbol for glyceraldehyde 3-phosphate dehydrogenase

GATA3 HGNC gene symbol for GATA binding protein 3

GC Glucocorticoid

GEP Gene Expression Profiling

GILZ Glucocorticoid-Induced Leucine Zipper protein; HGNC gene symbol: TSC22D3

GITR Glucocorticoid-Induced TNFR-Related protein; HGNC gene symbol: TNFRSF18

GLUT4 Glucose Transporter 4; HGNC gene symbol: SLC2A4

GMCSF Granulocyte-Macrophage Colony Stimulating Factor; HGNC gene symbol: CSF2

GO Gene Ontology

GR Glucocorticoid Receptor

GRE Glucocorticoid Responsive Elements

GSK3 Glycogen Synthase Kinase 3; HGNC gene symbols: GSK3A and GSK3B

GUS Glucuronidase Beta; HGNC gene symbol: GUSB

HCLS1 HGNC gene symbol for hematopoietic cell specific Lyn substrate 1

HGNC HUGO Gene Nomenclature Committee

HIP Hsp70-Interacting Protein; HGNC gene symbol: ST13

HOP Hsp70/Hsp90-Organizing Protein; HGNC gene symbol: STIP1

HSCs Hematopoietic Stem Cells

HSP40 Heat Shock Protein 40kDa

Abbreviations

HSP70 Heat Shock Protein 70kDa

HSP90 Heat Shock Protein 90kDa

IAPs Inhibitor of Apoptosis family of proteins

ICAM1 HGNC gene symbol for intercellular adhesion molecule 1

ICAM2 HGNC gene symbol for intercellular adhesion molecule 2

ICAM3 HGNC gene symbol for intercellular adhesion molecule 3

IFN γ Interferon Gamma; HGNC gene symbol: IFNG

IFIT2 HGNC gene symbol for interferon-induced protein with tetratricopeptide repeats2

Ig Immunoglobulin

IGHM Immunoglobulin Heavy Constant Mu

IGHG Immunoglobulin Heavy Constant Gamma

IGHV Immunoglobulin Heavy Variable

IGF1 HGNC gene symbol for insulin-like growth factor 1 (somatomedin C)

IL10 HGNC gene symbol for interleukin 10

IL12 HGNC gene symbol for interleukin 12

IL17 HGNC gene symbol for interleukin 17

IL1B HGNC gene symbol for interleukin 1, beta

IL2 HGNC gene symbol for interleukin 2

IL23 HGNC gene symbol for interleukin 23

IL6 HGNC gene symbol for interleukin 6

IL7R HGNC gene symbol for interleukin 7 receptor

IkB α Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; HGNC gene symbol: NFKBIA

IKK I κ B Kinase complex

IPA Ingenuity Pathways Analysis

IRF3 HGNC gene symbol for interferon regulatory factor 3

ITGAM HGNC gene symbol for integrin, alpha M (complement component 3 receptor 3 subunit)

JAK Janus Kinase family of tyrosine kinases

JNK c-Jun N-terminal Kinases family

KMO HGNC gene symbol for Kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)

LCK HGNC gene symbol for lymphocyte-specific protein tyrosine kinase

LPL HGNC gene symbol for lipoprotein lipase

LN Lymph Nodes

MCLL CLL case with mutated IGHV gene

WHO World Health Organization

MBL Monoclonal B cell Lymphocytosis

MAPK Mitogen Activated Protein Kinases family of proteins

MCL1 HGNC gene symbol for myeloid cell leukemia sequence 1 (BCL2-related)

MDM2 HGNC gene symbol for mdm2, p53 E3 ubiquitin protein ligase homolog (mouse)

MDR1 Multidrug Resistance protein 1; HGNC gene symbol: ABCB1

MEK MAPK/ERK Kinases or mitogen-activated protein kinase kinases

MHCII Major Histocompatibility Complex class II

MKP1 Dual specificity phosphatase 1; HGNC gene symbol: DUSP1

MYC HGNC gene symbol for v-myc myelocytomatosis viral oncogene homolog (avian)

MM Multiple Myeloma

mRNA messenger RNA

MURF1 Muscle-specific RING Finger protein 1, HGNC gene symbol: TRIM63

MYD88 HGNC gene symbol for myeloid differentiation primary response gene (88)

NFAT Nuclear Factor of Activated T-cells family of transcription factors

NFKB Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells

NHL Non-Hodgkin Lymphoma

NK Natural Killer

NOXA word in Latin for damage; HGNC gene symbol: PMAIP1

Abbreviations

NR3C1 HGNC gene symbol for nuclear receptor subfamily 3, group C, member 1

P23 Prostaglandin E Synthase 3 (cytosolic); HGNC gene symbol: PTGES3

PALM2-AKAP2 HGNC gene symbol for PALM2-AKAP2 readthrough

PARP Poly (ADP-Ribose) Polymerase family of proteins

PB Peripheral Blood

PBS Phosphate Buffered Saline solution

PBMC Peripheral Blood Mononuclear Cells

PCR Polymerase Chain Reaction

PE Phycoerythrin

PerCP-CyTM5.5 Peridinin chlorophyll protein-cyanin 5.5

PEPCK Phosphoenolpyruvate Carboxykinase; HGNC gene symbol: PCK2

PEST Peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T)

PI Propidium Iodide

PI3K Phosphatidylinositol 3-Kinases

PLA2 Phospholipases A2

PPID HGNC gene symbol for peptidylprolyl isomerase D; alias: cyclophilin 40

PTEN HGNC gene symbol for phosphatase and tensin homolog

PTP1B Protein-tyrosine phosphatase 1B; HGNC gene symbol: PTPN1

PUMA p53 Upregulated Modulator of Apoptosis; HGNC gene symbol: BBC3

QRT-PCR Quantitative Real Time Polymerase Chain Reaction

RAF Proto-oncogene serine/threonine-protein kinase; HGNC gene symbol: RAF1

RAFTK Related Adhesion Focal Tyrosine Kinase; HGNC gene symbol: PTK2B

RAS Small GTPase subfamily of proteins

RIN RNA Integrity Number

RPS6KB1 HGNC gene symbol for ribosomal protein S6 kinase, 70kDa, polypeptide 1

RT Room Temperature

SAMD9L HGNC gene symbol for sterile alpha motif domain containing 9-like

SD Standard Deviation

SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SF3B1 HGNC gene symbol splicing factor 3b, subunit 1, 155kDa

SHM Somatic Hypermutation

SLL Small Lymphocytic Lymphoma

SSC Side Scatter

STAT STAT family of transcription factors

SYK HGNC gene symbol for spleen tyrosine kinase

TBX21 HGNC gene symbol for T-box 21

TCR T Cell Receptor

TGF β Transforming Growth Factor beta

TMEM2 HGNC gene symbol for transmembrane protein 2

TNF α Tumor Necrosis Factor α ; HGNC gene symbol TNF

TP53 HGNC gene symbol for tumor protein p53

Tregs Regulatory T cells

UCLL CLL case with unmutated IGHV gene

VCAM1 HGNC gene symbol for vascular cell adhesion molecule 1

VLA-4 Very Late Antigen-4, integrin dimer composed by CD49d and CD29

XIAP HGNC gene symbol for X-linked inhibitor of apoptosis

ZAP70 HGNC gene symbol for zeta-chain (TCR) associated protein kinase 70kDa

Abbreviations

INTRODUCTION

1. CHRONIC LYMPHOCYTIC LEUKEMIA

1.1. Biological characteristics of Chronic Lymphocytic Leukemia

The World Health Organization (WHO) classification of hematopoietic neoplasias of 2008 describes the Chronic Lymphocytic Leukemia / Small Lymphocytic Lymphoma (CLL / SLL) as a lymphoproliferative disorder of small neoplastic B cell.¹ CLL occurs most frequently in persons older than 50 with a higher incidence in males.² In Western countries, CLL accounts for about 30% of all leukemias being the most frequent form of leukemia whereas in the Asian population it only constitutes 10% of all leukemias.³

The diagnostic criteria of CLL proposed by the WHO 2008 classification are the presence in peripheral blood (PB) of at least 5×10^9 B cells per liter with a monoclonal weak expression of one of the light chain immunoglobulin (Ig) genes. Also the lymphocytosis must persist for at least 3 months; nevertheless CLL diagnosis can be made with lower numbers of B cells when disease related symptoms are reported or when patients exhibit cytopenias. Frequently CLL cells are found simultaneously in PB, bone marrow (BM) and lymph nodes (LN). The SLL term is normally applied to non-leukemic forms, more precisely to cases with LN involvement, without cytopenias and with PB lymphocyte counts below 5×10^9 cells per liter.¹

Special attention should be given to the distinction between CLL and Monoclonal B cell Lymphocytosis (MBL), the latter also referring to cases of B cell monoclonal expansions in PB but with lymphocyte counts below 5×10^9 cells per liter and no lymphadenopathy, splenomegaly, hepatomegaly, cytopenias nor other type of symptoms.³ In the last years it has been hypothesized that MBL could be a precursor form of CLL since some MBL cases evolve to CLL at a rate of 1.1% of conversions per year.⁴

1.1.1. Morphological features

The CLL cells found on PB smears are characteristically small, mature, with a narrow border of cytoplasm, and a dense nucleus lacking discernible nucleoli and having a coarsely clumped chromatin (Figure 1). Many times, these typical CLL cells can be found mixed with cells presenting different features like cleaved cells, prolymphocytes, centrocytes, centroblasts and stimulated lymphocytes. Nevertheless, prolymphocytes only can represent up to 55% of the blood lymphocytes, otherwise, they will favor the diagnosis of B cell prolymphocytic leukemia. Another characteristic of CLL blood smears is the presence of Gümprecht nuclear shadows, or smudge cells, found as cell debris due to the fragile nature of CLL cells.¹

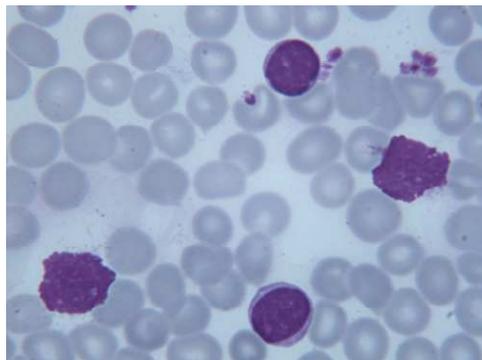


Figure 1. Morphological characteristics of CLL cells found in peripheral blood

Typical CLL cells and Gümprecht nuclear shadows are shown.

In CLL, the BM infiltration is a common feature and normally CLL cells represent more than 30% of the total cell counts of BM aspirates. The BM CLL cells present the same morphologic characteristics of those described in PB. It is also important to mention that although BM aspirates or biopsies are not required for the diagnosis of CLL, the histological pattern of bone marrow infiltration was shown to have a prognostic value. On the other hand, it is recommended to perform a BM study before the onset of therapy.⁵

The patterns of BM involvement described in CLL are interstitial, nodular, diffuse and mixed.⁶ In the interstitial pattern, CLL cells infiltrate the BM in between the fat cells without affecting the normal BM architecture. The nodular pattern is the less frequent and it is characterized by the presence of nodules of small lymphocytes replacing the normal hematopoietic cells and the fat cells. The diffuse pattern is characterized by the complete destruction of BM architecture since CLL cells massively replace the normal hematopoietic cells and the fat cells. Finally, the mixed pattern is the combination of the interstitial and nodular infiltration. Mixed pattern and diffuse pattern are observed in patients with short survival.⁶

CLL can infiltrate LN, its pattern of infiltration is denominated pseudofollicular since pale areas on a dark background are observed. These pale areas are proliferation centers constituted by small to medium size cells, prolymphocytes, and by large cells called paraimmunoblasts. The dark background is formed by the typical small CLL cells. The mitotic activity of the CLL cells in LN is usually very low except in the pale areas.¹

The involvement of spleen by CLL is normally confined to the white pulp; nevertheless, red pulp can also be affected. As in LN, proliferation centers can be observed.¹

1.1.2. Immunophenotypic features

The CLL cells express pan B antigens like CD19, CD20, CD22 and CD79a on their surface. With the exception of CD19, the expression of these pan B antigens is characteristically dim when compared to normal B cells. The membrane expression of the immunoglobulin genes is also weak and normally CLL cells express IgM and IgD. In rare occasions, class switch occurs and CLL cells express IgG.⁷

Unlike normal B cells, CLL cells aberrantly co-express the T cell antigen CD5, as well as the CD23 and the CD43 antigens. Moreover CLL cells lack CD10 expression and usually lack the expression of FMC7, CD79b and CCND1 which are important features to distinguish CLL from the remaining lymphoproliferative disorders. Notwithstanding, the presentation of CLL cells not always accomplish the described immunophenotypic criteria.¹

1.1.3. Genetic and molecular characteristics

The pathogenic mechanisms of CLL are a subject of intense research and at the present multiple facets have been disclosed like genetic aberrations, antigen drive and other microenvironment interactions.

The molecular and genetic pathogenesis of CLL is unknown. The most frequently cytogenetic abnormalities found in CLL are the deletions of chromosomal regions in 13q14 (55%), 11q22-q23 (18%), 17p13 (7%), and 6q21 (6%) and the trisomy of chromosome 12 (16%).⁸ Interestingly, in CLL, genetic abnormalities do not involve the heavy and light chain loci of the immunoglobulin gene as observed in other B cell non-Hodgkin lymphomas (NHL). The genetic lesions of CLL were found to be clinically relevant since their presence at the time of diagnosis or their acquisition during the course of the disease, are correlated with survival and resistance to treatment.^{8;9} The study of the chromosomal loci affected by the genetic lesions allowed the identification of the genes involved: ataxia telangiectasia mutated (ATM) gene in chromosomal region 11q22-q23, TP53 in 17p13, and the micro-RNA genes MIR15A and MIR16-1 in 13q14.¹⁰⁻¹²

The 13q14 chromosomal deletions in CLL are associated with the down-regulation of miR-15a and miR-16-1 and in mice models it has been reported that abnormalities in the MIR16 locus are related to B cell clonal proliferations with CLL

features.¹³ Moreover, a recent work has shown that the DLEU2 / MIR15A / MIR16-1 cluster controls B cell proliferation and that its deletion leads to CLL.¹⁴

Deletions in 11q22-q23 almost invariably comprise ATM which is implicated in the repair of DNA damage.¹⁵ As so, ATM-deficiency is thought to contribute to the CLL pathogenesis since it permits the accumulation of additional genetic lesions.

In CLL, the 17p13 chromosomal deletions always include the TP53 suppressor gene. 80 to 90% of the patients with deletion of one copy of the TP53 locus have a mutation in TP53 in the remaining copy, thus implying that almost all patients with CLL with 17p13 deletion have a non functional p53 pathway.¹⁶ In patients with CLL it has been reported that, at diagnosis, the incidence of 17p13 deletions is 5% whereas the incidence of TP53 mutations is 10%. Of these 10%, around 4.5% have TP53 mutations without 17p13 deletions.¹⁷⁻¹⁹ More importantly, both mutations in TP53 and 17p13 deletions have adverse prognostication value.¹⁷⁻¹⁹ The importance of p53 function is based on the fact that many chemotherapy agents produce DNA damage, thus inducing apoptosis in a p53 dependent manner (Figure 2).²⁰

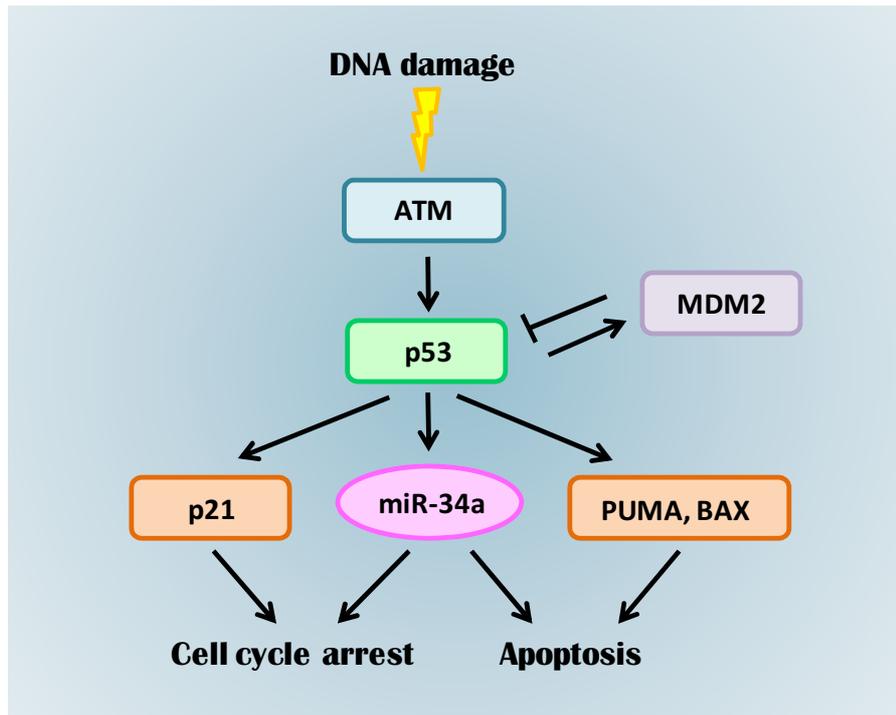


Figure 2. DNA damage and p53 pathway (Adapted from¹⁰)

Interestingly, a regulator of p53, MDM2, is located on chromosome 12. MDM2 impairs p53 functions because it abrogates p53 transcriptional activity,²¹ and because it promotes p53 degradation in the proteasome.²² It has been hypothesized that CLL cases with trisomy 12 may show low levels of p53 expression, but since these cases have high CD20 expression, they present favorable overall survival in the Rituximab era.¹⁰ The MIR34A gene that is located on the chromosomal regions 1p36 and 11q23 and miR-34a has been shown to mediate some of the actions of p53 after the induction of DNA damage.²³ In summary, not only 17p13 deletions but also other cytogenetic abnormalities found in CLL cells seem to play a role in the apoptosis mediated by the p53 pathway.

In CLL, the presence of 17p13 deletions and TP53 mutations is dramatically increased in the refractory and relapsed patients most probably due to the selection of the 17p13 deleted and TP53 mutated cells.¹⁰ A report on patients refractory to fludarabine showed that 44% of the patients had 17p13 deletion and / or TP53 mutation: 25% of the patients had both abnormalities, 12% presented mutations in

TP53 only, and 7% had the 17p13 deletion solely.²⁴ The identification of 17p13 deletion (and TP53 mutation) is of major importance, and nowadays the treatment approaches for this patient group relies on non-genotoxic drugs like alemtuzumab, flavopiridol, lenalidomide, or glucocorticoids, alone or in combination with monoclonal antibodies.²⁵

As for most of the other malignant diseases, molecular biology has made possible the translation to clinical practice of recurrent observations with prognostic value for treatment approach. Technological advances continue to disclose new gene abnormalities in CLL. For example, recent works using next-generation sequencing analysis for whole genome sequencing have identified recurrent mutations in some genes, those being the most prevalent found in NOTCH1 (12.2% of patients with CLL),²⁶ SF3B1 (9.7 to 15% of patients with CLL),^{27;28} and MYD88 (2.9% of patients with CLL).²⁶

Mutations in NOTCH1 affect the functionality of the PEST domain leading to the accumulation of the protein and increasing signaling of NOTCH1 pathways.²⁶ Moreover, NOTCH1 mutations were found to be associated with clinically aggressive forms of CLL,²⁹ and they are an independent predictor of overall survival.³⁰ SF3B1 encodes a splicing factor and the mutations in this gene presenting in CLL cells have been shown to lead to altered splicing function thus pointing pre-mRNA splicing as a critical cellular process contributing to disease development.²⁸ MYD88 codifies for a protein involved in the signaling through IL1R and Toll-like receptors.³¹ Apparently, the MYD88 mutation found in CLL leads to increased secretion of cytokines responsible for the recruitment of macrophages and T lymphocytes, a milieu that favors CLL cells survival.²⁶ Interestingly the patients with CLL with MYD88 mutation are diagnosed at young ages as well as in advanced clinical stages.

Previous molecular biology studies like those of whole gene expression profiling (GEP) have provided data for a better understanding of CLL biology. Klein and colleagues have shown that the GEP of CLL cells was similar to that of mature B cells.³² Since that, CLL has been viewed as a malignancy originated from the

oncogenic transformation of a common cellular precursor that resembles an antigen experienced B cell. Moreover, it has been observed that CLL cells express B cell receptors (BCRs) with evidence of antigen experience, and more notably, BCRs from different individuals are homologous in their antigen binding regions.^{33;34} These findings allowed the delineation of subsets of stereotyped receptors and strengthens the notion that antigens play a critical role in pathogenesis of CLL.³⁵⁻³⁷

In the past, CLL was thought to be an accumulative disease and a consequence of a defect in the cell apoptosis machinery. The quiescent appearance of the CLL cells, their small size and condensed chromatin, and the lack of mitosis is evidence which sustains this theory. From a clinical standpoint CLL is considered an indolent disease, since the reported median survival of patients is around 10 years and disease treatment is only needed when the accumulation of cells compromises the life of the patient.^{38;39} Nevertheless, it is known that in some patients the disease course is aggressive and studies have shown significant levels of proliferation.^{40;41}

In the last years, there was a resurgent interest in CLL proliferative rates. Experiments using deuterium water or glucose have demonstrated a correlation between birth rates and disease activity, pointing out that proliferation seems to exist.^{42;43} Moreover, studies measuring the telomere length of CLL cells have shown that they were much shorter than those of B cells of age-matched normal donors, and that they were shorter in the CLL subgroup with worst prognosis according to the mutational status of the immunoglobulin heavy variable (IGHV) genes.^{44;45} Telomerase activity is known to be higher in the germinal center, corroborating that the aggressive clinic behavior of some patients with CLL must be due to an increase proliferative activity of its cells.

Recently, the proliferation rate of CLL cells was shown to be different according to particular phenotypes; CD38 positive, CD5 bright, and CXCR4 dim cell populations showed higher proliferation rates than those CD38 negative, CD5 dim, and CXCR4 bright.^{46;47} The results of these studies pointed to the existence of two subsets within

the leukemic clone, one corresponding to cells recently emigrating from the germinal centers and the other corresponding to resting cells. Moreover, they support the reasoning that extracellular signals are playing an important role in the proliferation and cell death of CLL cells.

Altogether, data has shown that the microenvironment plays an important role in CLL cell fate through the activation of signalling pathways, namely through BCR, Toll-like receptors, cytokine receptors, and chemokines receptors.

Finally, it is important to mention the interesting results of a recent work on CLL hematopoietic stem cells (HSCs). The existence of CLL HSCs have always been underscored since the CLL cells present BCR clonality suggesting that the lymphomagenic events followed VDJ recombination. Kikushige and colleagues have successfully engrafted immunodeficient mice with HSCs obtained from patients with CLL and these mice developed monoclonal or oligoclonal B cells simulating MBL.⁴⁸ MBL is thought to be the precursor phase of CLL, and the chromosome alterations found in CLL are probably the secondary events needed for disease development. CLL HSCs must accumulate oncogenic events like genetic and / or epigenetic mutations that are further responsible for their aberrant behavior. The results of the above work not only changed the knowledge of CLL biology but also support the lack of benefit of autologous stem cell transplantation in patients with CLL.

1.2. Prognostic markers in Chronic Lymphocytic Leukemia

The clinical course of CLL is heterogeneous, whereas most of the patients will not need therapy for years, others will eventually die due to disease related complications.^{38;39} Importantly, CLL remains an incurable disease, and treatment decisions require the assessment of the risk for each patient. The onset of treatment is usually based on the presence of active disease, although some patients would probably benefit from having earlier treatment. Thus, there is a need to identify clinical and biological features that allow the identification of patients prone to develop an aggressive form of the disease.

Clinical stages given by Rai and Binet systems are still considered the most important for prognostication, since they have been tested in many and large CLL series (see Table 1).^{49;50}

Table 1. Clinical stages of CLL according to Rai and Binet systems

Stage system	Low risk	Intermediate risk		High risk	
Binet	A Hb \geq 10 g/dL Platelets \geq $100 \times 10^9/L$ \leq 2 sites involved *	B Hb \geq 10 g/dL Platelets \geq $100 \times 10^9/L$ > 2 sites involved *		C Hb < 10 g/dL or Platelets < $100 \times 10^9/L$	
Rai	0 Lymphocytosis only	I Lymphocytosis and Lymphadenopathy	II Lymphocytosis and Splenomegaly and / or Hepatomegaly	III Lymphocytosis and Hb < 11g/dL	IV Lymphocytosis and Platelets < $100 \times 10^9/L$

*Sites involved are liver, spleen, lymph nodes (either unilateral or bilateral) in inguinal, axillary and cervical regions.

Both systems take into account the blood lymphocyte count, platelets count, hemoglobin levels, organomegaly, and lymphoid areas involved. Advanced clinical stages III-IV / C (high risk) show fast progression and median survival of 4 years

whereas stages 0 / A (low risk) and I-II / B (intermediate risk) show variable evolution. Due to the performance of blood analyses for routine purposes, nowadays more than 80% of the cases of CLL are diagnosed in asymptomatic and early stage forms. Unfortunately, clinical stages according to Rai and Binet systems are not useful to identify those patients in early stage that are likely to progress. For these reasons, during the last 10 years, new prognostic markers have been identified, along with the classical ones, in order to predict the outcome of the patients with CLL.

Classical prognostic variables include age, sex, performance status,⁵¹ blood lymphocyte count, lymphocyte morphology in PB, blood lymphocyte doubling time, and BM infiltration pattern. In addition, some biological features have been added to the prognostic armamentarium: serum levels of lactate dehydrogenase, β -2 microglobulin, sCD23, and thymidine-kinase. It is important to mention that blood lymphocyte counts higher than $50 \times 10^9/L$, blood lymphocyte doubling time lower than 12 months, and diffuse BM infiltration pattern were found to have an adverse impact in time to treatment and survival.²

Attempts have been made in order to create a prognostic scoring system in CLL⁵²⁻⁵⁴ as the ones applied in diffuse large B cell lymphoma (International Prognosis Index, IPI), in follicular lymphoma (Follicular Lymphoma International Prognosis Index, FLIPI), or in mantle cell lymphoma (Mantle Cell Lymphoma International Prognosis Index, MIPI). Nevertheless, no consensus has been reached so far and further studies are needed to validate and standardize the parameters to be used in the routine management of the patients with CLL.

Some biological prognostic markers were identified to be useful in predicting disease free survival and overall survival in early stage CLL. The most extensively studied are the mutational status of the IGHV genes,^{55;56} the immunophenotypic markers ZAP70 and CD38,⁵⁶⁻⁵⁸ and the cytogenetic abnormalities.⁸

In the 1990's, two papers written by Chiorazzi *et al.* and by Stevenson *et al.* disclosed that patients with CLL with unmutated IGHV genes have unfavorable

biological features in addition with a rapid clinical progression, treatment requirement, and short survival.^{55;56} Conversely, they showed that patients with mutated IGHV genes have favorable clinic and biological features, do not require therapy for long periods of time, and have a long survival. Importantly, the mutational status of the IGHV genes has prognostic value in patients with early stages of disease and does not change during the clinical course of the disease.

It has been shown that the usage of the IGHV3-21 gene has a poor prognostic value independently of the mutational status.⁵⁹ Further works demonstrated that not only V gene usage, but the configuration of the CDR3 of heavy chains, had prognostic implications and this is sometimes independent of the mutational status.^{36;37;60} An active line of research has been opened on IGHV genes usage, mutational load, and prognostic impact.

Along with the identification of the prognostic value of the mutational status of the IGHV genes, Damble *et al.* have demonstrated that the expression of CD38 was correlated with the unmutated status of the IGHV genes and with shorter survival.⁵⁶ Later, other studies have shown that although CD38 expression has independent prognostic value, this fact does not correlate with the mutational status of the IGHV genes, and that its expression changes during the course of disease.⁶¹⁻⁶³ However, it is now accepted that CD38 expression is an independent prognostic marker in CLL.^{64;65}

Studies on the molecular characteristics of CLL like the GEP studies provided evidence for the discovery of ZAP70 as an important prognostic marker.⁶⁶ ZAP70 was shown to be a surrogate marker of IGHV mutational status, since ZAP70 expression and the unmutated status of the IGHV gene had an excellent correlation.^{57;58} Later, the independent prognostic value of ZAP70 was unveiled.^{67;68} Unlike CD38, ZAP70 expression remains stable in time, and it also can be determined by flow cytometry. Efforts are currently being made to standardize the assessment of ZAP70 expression by flow cytometry (http://www.ericll.org/projects/ZAP70_CD38_harmonization.php). The International Workshop on CLL guidelines recommended ZAP70 expression

determination in clinical trials.⁵ Of note, ZAP70, CD38, and IGHV mutational status should not yet be taken into consideration for treatment decisions, as further studies are needed.

Cytogenetic abnormalities can be detected by interphase fluorescent *in situ* hybridization (FISH) in more than 80% of all CLL cases,⁸ and FISH studies are a current practice in the diagnosis and follow-up of CLL. The cytogenetic abnormalities most frequently found in CLL have independent prognostic relevance. As first reported by Dohner *et al.*, deletion in 13q14 as sole aberration is associated with long overall survival; on opposite, deletion in 11q22-q23 and particularly those in 17p13 are associated with short overall survival.⁸ In addition, CLL cases with trisomy 12 or cases without the most frequently cytogenetic abnormalities found in CLL have intermediate overall survival. This allowed the construction of a hierarchical model for the prognostic impact of cytogenetic abnormalities in CLL: deletion 17p13 > deletion 11q22-q23 not including 17p13 deletion > trisomy 12 not including 17p13 deletion and 11q22-q23 deletion > no cytogenetic abnormalities > deletion 13q14 not including 17p13 deletion, 11q22-q23 deletion and trisomy 12 (descending order of adversity). Interestingly, the results of a clinical trial in the Rituximab era have shown that the presence of 11q22-q23 deletion and trisomy 12 has been associated to a better progression free survival than the absence of cytogenetic abnormalities.⁶⁹

The prognostic impact of the cytogenetic abnormalities was shown to be independent of the mutational status of the IGHV gene.^{70;71} Interestingly, clonal evolution occurs more frequently in patients with unmutated IGHV genes and ZAP70 expression.^{72;73} Most importantly, cytogenetic abnormalities are the only prognostic markers with demonstrated importance for treatment decisions, namely the deletion in 17p13.⁵ Evidence sustained the inefficiency of fludarabine or alkylating based therapies in this setting. The standard CLL therapy, Rituximab / fludarabine / cyclophosphamide, shows very poor responses in the subgroup of patients with 17p13 deletion: 68% of overall response rate and 5% of complete response.⁶⁹ Thus, patients with CLL

presenting 17p13 deletion should be considered for alternative therapies and ultimately for allogenic stem cell transplantation.²⁵

Several other protein and gene levels have been further correlated with IGHV genes mutational status, though they all have the independent capacity to predict prognosis. These include, among many others, lipoprotein lipase (LPL) gene alone or LPL/ADAM29 genes ratio,⁷⁴⁻⁷⁶ integrin alpha 4 (CD49d) protein,⁷⁷ HCLS1 protein,⁷⁸ CLLU1 gene,⁷⁹ and FCRL2 gene.^{80;81}

The research on prognostic markers in CLL is intense, and with the introduction of immunochemotherapy schedules, many of the former markers have to be confirmed. In the future, new ones will probably arise and will allow a better management of patients with CLL. Importantly, biological features of the patients can be related to drug responses. As so, other characteristics like the presence of certain cell receptors and proteins could be correlated with treatment responses and thus be used in treatment decisions.

1.2.1. The immunoglobulin heavy variable genes and their mutational status analysis

The immunoglobulin is a part of the BCR which allows B cells to recognize foreign antigens. Immunoglobulins are composed of two identical heavy chains and two identical light chains. Functionally there are two main regions to be considered, the N-terminal or variable domain, responsible for the antigen recognition, and the C-terminal or constant domain, with effector properties. The variable domains of the heavy chains are codified by 3 different types of genes, namely: V, D and J genes and the variable domains of the light chains are codified by the V and J genes. In both heavy and light chains, the limited repertoire of these genes are randomly assembled by DNA rearrangement giving rise to an enormous variety of immunoglobulins.⁸² In the variable

domains there are 4 relatively conserved framework regions (FR1 to 4) interspersed by 3 highly variable regions called CDR (CDR1 to 3).⁸³ FRs are responsible for maintaining the structure of the domain and CDR regions directly interact with antigens, CDR3 being the most important determinant of antigen specificity as well as the most variable, since it is located at the junction of the V, D, and J genes.

Another event responsible for the large diversity of immunoglobulins is the somatic hypermutation (SHM) process. Mature B cells can be stimulated by antigens through their BCR that, together with other microenvironment stimulus, lead to the organization of specialized structures called germinal centers in the secondary follicles of peripheral lymphoid tissues.⁸⁴ The SHM essentially takes place in germinal centers and is mediated by activation-induced cytidine deaminase. Basically, it consists in single base substitutions affecting the rearranged VDJ genes, sparing the constant domain. The mutations can be silent or result in the replacement of an aminoacid, and can occur both in FR and CDR. Typically, replacement mutations tend to localize in CDR thus increasing the antigen affinity. On the other hand in FR, they are counter-selected since they would affect the overall structure of the domain, and as a consequence, enrichment in silent mutations is observed.^{85;86} In rare occasions, the SHM may introduce insertions (duplications of a neighboring nucleotide or sequence) or deletions within immunoglobulin rearranged sequences.⁸⁷

The study of the mutational status of the immunoglobulin genes may help to identify the origin of lymphoid malignancies along with the B cell differentiation pathway. Also, the mutation status of the immunoglobulin genes is a powerful prognostic marker in CLL as explained before. Traditionally, the analysis of the mutational status is confined to the IGHV and using the arbitrary cut-off value of 98% in homology to the germline IGHV gene. It has been found that around 40% of patients with CLL carry mutated IGHV genes.^{88;89} It is important to mention that although the mutational status of the IGHV genes does not change during clinical course, the use of the 98% cut-off may not reflect the real impact of mutations. In some instances, few or

even only a single nucleotide mutation can be introduced by SHM, and thus, it should be considered a real mutation.³⁷ These circumstances may lead to incorrectly assigned unmutated IGHV genes.

1.2.2. ZAP70 expression

ZAP70 is a tyrosine kinase of the Syk family initially isolated in T and natural killer (NK) cells where it plays a crucial role in the proximal signaling of T and NK cell receptors respectively.⁹⁰ More recently, ZAP70 expression has been reported in normal B cell precursors and in some subsets of activated B cells.^{91;92} ZAP70 expression has been also detected in some cases of B cell proliferative diseases like CLL, B acute lymphoblastic leukemia (ALL) and Burkitt lymphoma.^{57;92;93}

The importance of ZAP70 expression in CLL was disclosed in 2003 when it was found a correlation between the mutational status of the IGHV genes and the expression of ZAP70.^{57;58} The concordance between these two features is around 75-95% depending on the report.^{57;58;67;94} Later, studies have demonstrated that ZAP70 had a prognostic value of its own; ZAP70 expression levels allow the discrimination of patients in two groups with different prognosis. Patients with CLL who have high ZAP70 expression ($\geq 20\%$ positive CLL cells) have inferior overall survival,^{57;95;96} and have shorter time to progression or treatment.^{57;58} In addition, the high expression of ZAP70 was associated with a faster reappearance of detectable minimal residual disease and with a faster progression after immunochemotherapy.⁹⁷

Studies have been performed in order to address the biological role of ZAP70 in CLL cells. It has been found that CLL cells with high ZAP70 expression had increased signaling through BCR.⁹⁸ Moreover, ZAP70 expression has been associated with

increased ability to respond to migratory and survival signals.⁹⁹ In this line, a recent report has proved the direct implication of ZAP70 in the migration induced by CCL21.¹⁰⁰

In summary, in CLL cells, the high ZAP70 expression is associated with adverse biological features like unmutated IGHV genes and high CD38 expression, and is correlated with a poor clinical outcome. Importantly, ZAP70 expression can be easily determined by flow cytometry, and it retains prognostic value regarding time to progression in untreated stage A patients.

2. CORTICOSTEROIDS

2.1. Classification: glucocorticoids and mineralocorticoids

Corticosteroids are a class of compounds including both the steroid hormones produced in the adrenal cortex of vertebrates (endogenous corticosteroids) and the synthetic analogues of these hormones (synthetic corticosteroids). The synthesis of corticosteroids in the adrenal cortex is made from cholesterol and is controlled by the adrenocorticotrophic hormone through long series of enzymatic mechanisms involving many oxidation reactions.¹⁰¹ The endogenous corticosteroids have 19 carbon atoms and show both mineralocorticoid and glucocorticoid activities.¹⁰² In its sense, the glucocorticoid activity is the corticosteroids role in the regulation of the glucose metabolism. On the other hand, the mineralocorticoid activity is the ability of corticosteroids to regulate the transport of ions.

The corticosteroids activities are explain by the existence of two different steroid receptors, namely the glucocorticoid receptor (GR) and the mineralocorticoid receptor.¹⁰³ Endogenous corticosteroids can bind both receptors thereby having overlapped glucocorticoid and mineralocorticoid activities. The power of each activity depends on the affinity of the glucocorticoid receptor and of the mineralocorticoid receptor for a particular corticosteroid. For example, if the affinity of the glucocorticoid receptor is very high and the affinity of the mineralocorticoid receptor is low, then the glucocorticoid activity will prevail. Endogenous corticosteroids normally bind strongly to one of the receptors and this was used to classified corticosteroids in glucocorticoids and mineralocorticoids. There are no pure endogenous glucocorticoids or mineralocorticoids, but synthetic corticosteroids were developed in order to increase the mineralocorticoid or the glucocorticoid activity, or even to abrogate one of them. These are the cases of dexamethasone and betamethasone that only show

glucocorticoid activity. Usually, the endogenous cortisol (or the synthetic analogue hydrocortisone) is used as a standard to calculate the glucocorticoid and mineralocorticoid activity of the different corticosteroids (Table 2).

Table 2. Glucocorticoid and mineralocorticoid activity of the most used corticosteroids

Name	Glucocorticoid activity	Mineralocorticoid activity
Cortisol (hydrocortisone)	1	1
Prednisone	3.5-5	0.8
Prednisolone	4	0.8
Methylprednisolone	5-7.5	0.5
Dexamethasone	25-80	0
Betamethasone	25-30	0
Triamcinolone	5	0
Fludocortisone acetate	15	200
Deoxycorticosterone acetate (DOCA)	0	20
Aldosterone	0.3	200-1000

Glucocorticoids like methylprednisolone and dexamethasone are known to have antileukemic effects on CLL cells being both broadly used. Dexamethasone has higher anti-inflammatory activity than methylprednisolone but both drugs show similar anti-proliferative and apoptotic effects.¹⁰⁴ The concentration of glucocorticoid necessary to obtain 50% of the maximal apoptotic effect (EC50) has been determined in previous studies and it is 10^{-7} M for methylprednisolone and between 10^{-8} and 10^{-7} M for dexamethasone.¹⁰⁴ Thus, dexamethasone and methylprednisolone have equivalent antileukemic effects and can be indistinctively administered to patients with CLL.

2.2. Molecular basis of glucocorticoid action

Glucocorticoids are lipophilic and for this reason they are transported in the blood in a reversible complex with proteins. Around 90% of the cortisol found in blood is bound to the corticosteroid binding globulin (CBG) and it is generally accepted that the cortisol bound to CBG had a restricted access to target cells being active only the free cortisol.¹⁰⁵ Glucocorticoids passively diffuse across the plasma membrane into the cell cytoplasm where they encountered the GR.¹⁰⁶ However, evidence pointed towards an active role of CBG in glucocorticoid action through the binding of CBG-glucocorticoid complexes to cell membranes.¹⁰⁷

The GR is a member of the steroid hormone receptor family of proteins and its gene, NR3C1, is localized on chromosome 5q31-32. NR3C1 gene originates different transcript variants because it has alternative sites for the initiation of transcription, and because alternative splicing of mRNA occurs. Additional diversity in GR is due to post-translational modifications like phosphorylation, ubiquitination, and sumoylation.¹⁰⁸ There are several GR variants such as GR α , GR β , GR γ , GR-A, and GR-P, and they are expressed at different ratios in distinct cell types. The GR variants have been shown to be functionally different since they display diverse cytoplasm-to-nucleus trafficking patterns and distinct transcriptional activities. The major functional variant is the full length GR α and it consists of a N-terminal transactivation domain, a DNA binding domain with two zinc finger motifs, a hinge region, and a C-terminal ligand binding domain.¹⁰⁹

The GR resides in the cytoplasm forming a complex with co-chaperone proteins like heat shock proteins such as HSP90 and HSP70, and immunophilins like FKBP4, FKBP5, and PPID. These co-chaperones are inter-exchangeable and determine the conformation of the GR as well as its nuclear translocation.¹¹⁰ A model for hormonal activation of the GR was proposed.¹¹¹ In the cytoplasm and in the absence of glucocorticoids, the GR is mainly bound to FKBP5. The ligation of the glucocorticoid to

the GR causes the switch of FKBP5 by FKBP4. FKBP4 unlike FKBP5 has the ability to interact with dynein, a motor protein that furthers translocate the glucocorticoid-GR-complex to the nucleus. Once in the nucleus, this complex is able to trigger genomic effects by activating or repressing gene transcription. It can dimerize and bind to palindromic elements of the promoter region of target genes called glucocorticoid responsive elements (GRE), or as a monomer, it can interact with transcription factors already bound to the DNA.

The ligation of the glucocorticoid-GR-complex to GRE in general activates gene transcription through the recruitment of co-activator proteins like histone acetyltransferases.¹¹² Although, the glucocorticoid-GR-complex can bind to negative-GRE and can abrogate gene transcription through the recruitment of co-repressor proteins like histone deacetylases. Moreover, the glucocorticoid-GR complex can bind to composite GRE. These types of GRE bind complexes composed by the glucocorticoid-GR and transcription factors. The ligation to composite GRE can either induce or inhibit gene transcription depending on the type of composite GRE. In addition, the glucocorticoid-GR complex can modulate gene transcription by interaction with transcription factors already bound to DNA, a process known as tethering. Depending on the transcription factor, the net result can either be the activation or the repression of transcription (Figure 3).¹¹²

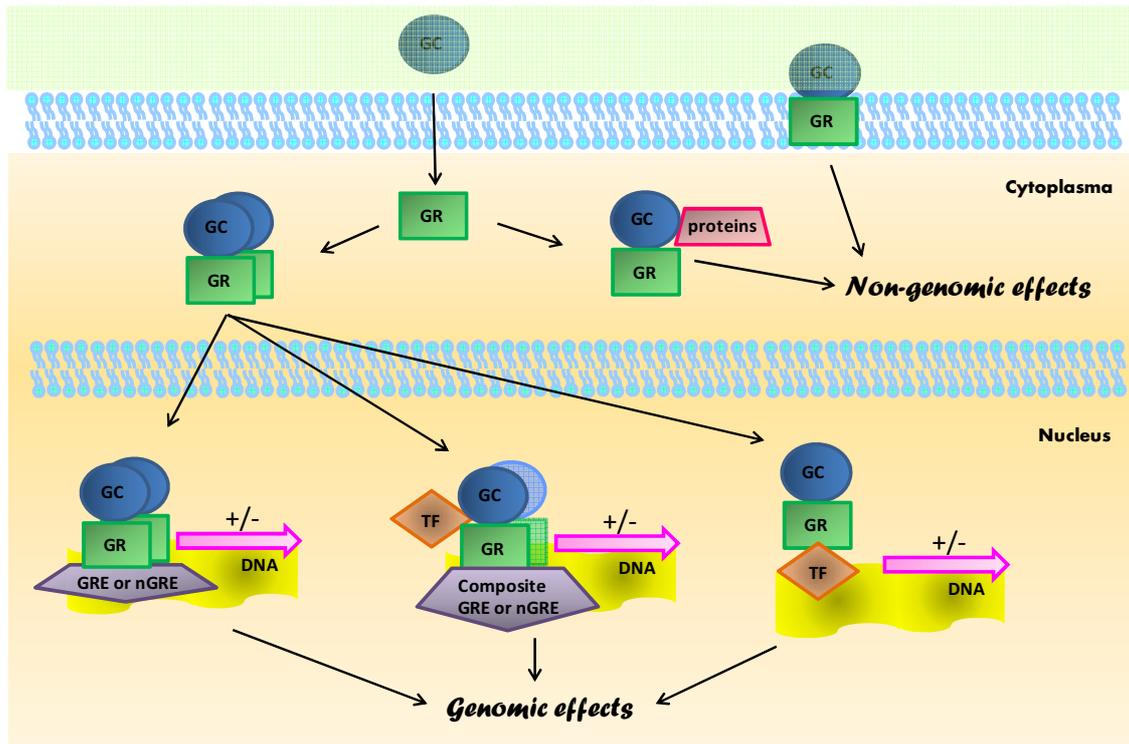


Figure 3. Molecular mechanisms of action of glucocorticoids

GC- glucocorticoid; GR- glucocorticoid receptor; GRE- glucocorticoid responsive element TF- transcription factor.

Furthermore, glucocorticoids are able to produce cellular responses within minutes independently of *de novo* gene transcription, the so called non-genomic effects. Two mechanisms have been reported, one is mediated by the ligation of the glucocorticoid to GRs present in the cell membranes. The T cell receptor (TCR) is associated to membrane bound GR and once glucocorticoids bind GR, the association between the TCR and the GR is disrupted and signaling through the TCR is abrogated.¹¹² The other mechanism occurs in the cytoplasm and is due to the direct protein-protein interaction between the glucocorticoid-GR complex and proteins such as c-Jun N-terminal kinases (JNK), phosphatidylinositol 3-kinases (PI3K) or AKT.¹¹³

2.3. Glucocorticoid physiological versus pharmacological activity

Early studies on glucocorticoids in the 1930s were focused on their physiological role since they were found to enhance and mediate response to stress. Nevertheless in 1949, it was reported that glucocorticoids could also protect cells from exacerbated responses to stress, unveiling their anti-inflammatory action.¹¹⁴ Since then, the study of the glucocorticoids was redirected to their pharmacological properties. The dual behavior of glucocorticoids was difficult to interpret at the moment but nowadays it is known that this dichotomy depends on the type of receptor involved, on the concentration of the glucocorticoid, and on the time of exposure.¹¹⁵ However, it turns to be one of the major difficulties of glucocorticoids use in therapy, and side effects arise from the unwished interference in physiologic homeostasis. Importantly, different synthetic glucocorticoids were shown to induce different GR conformations and thus to have different gene regulatory properties. This has allowed the design of glucocorticoids that have the beneficial anti-inflammatory effects and few or none of the unwanted metabolic effects.

2.3.1. Physiological effects of glucocorticoids

Glucocorticoids have physiological effects since they control the metabolism of carbohydrates, proteins, and lipids, as well as the balance of calcium. Glucocorticoids induce glucose formation by different ways (Figure 4). They inhibit glucose uptake in fat and muscle cells by inhibiting several steps of the insulin signaling cascade and, by impairing the translocation of the glucose transporter GLUT4 from the intracellular vesicles to the cell surface.¹¹⁶ They also increase gluconeogenesis in liver and muscle cells. In the liver they induce the synthesis of enzymes involved in the gluconeogenesis like PEPCK and G6PC.¹¹⁷

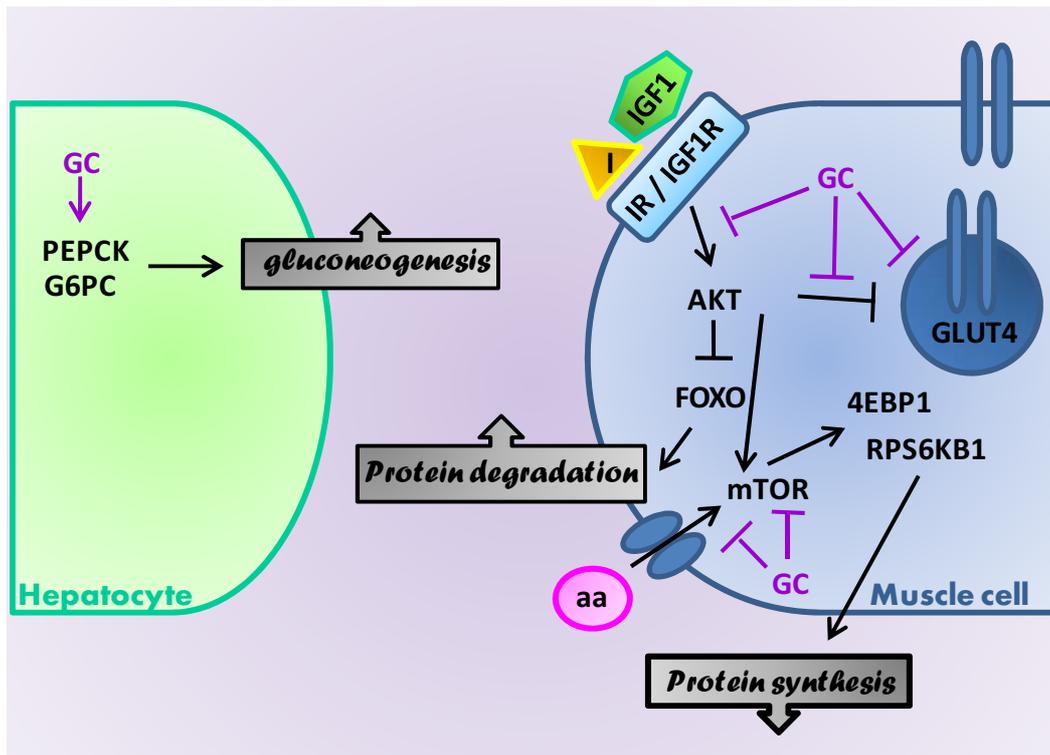


Figure 4. Physiological effects of the glucocorticoids in the metabolism of glucose and proteins

aa- amino acid; GC- glucocorticoid; I- insulin; IR- insulin receptor.

Glucocorticoids interfere in the metabolism of proteins; they decrease the rate of protein synthesis, and they increase the rate of protein breakdown (Figure 4.).¹¹⁸ Glucocorticoids impair protein synthesis by several ways; they reduce the transport of amino acids into the muscle, and they inhibit the anabolic effects of insulin and of insulin-like growth factor 1 (IGF1). They also reduce the transport of amino acids to the cells. Moreover, through the inhibition of the AKT/mTOR cell signaling pathway glucocorticoids impair the activation of protein synthesis mediators like the translation initiation factor 4E binding protein 1 (4EBP1) and the ribosomal protein S6 kinase 1 (RPS6KB1).

On the other hand, glucocorticoids induce proteolysis through the activation of proteolytic systems like the ubiquitin-proteasome system.¹¹⁸ They activate MURF1 and FBXO32, two proteins of the ubiquitin-proteasome system. Glucocorticoids also

upregulate the expression of the transcription factors FOXO1 and FOXO3, which are thought to play a pivotal role in the ubiquitin-proteasome pathway.

Glucocorticoids increase the amount of fatty acids in circulation through the hydrolysis of circulating triglycerides by lipoprotein lipase. Subsequently, fatty acids are available to muscle cells, adipocytes, and hepatocytes.¹¹⁹ Glucocorticoids increase *de novo* lipid production in hepatocytes since they induce the expression of fatty acid synthase. Moreover, they regulate the metabolism of the adipose tissue and the differentiation of pre-adipocytes into mature adipocytes. The glucocorticoids facilitate lipolysis by inducing lipase expression as well as other lipolysis mediators.

Glucocorticoids also trigger effects on the phosphor-calcium mechanism.¹¹⁵ They decrease the intestinal absorption of calcium, and they promote the excretion of calcium in the kidney. Thus, they accelerate the negative calcium balance which induces osteoporosis.

Unfortunately, many of the glucocorticoids effects in the metabolism turn out to be the major problem of chronic treatments. Glucocorticoids are responsible among others for central adiposity, hepatic steatosis, dyslipidemia, muscle mass atrophy, insulin resistance, glucose intolerance, and in extreme situations, for the diabetes onset.¹²⁰

2.3.2. Pharmacologic effects of glucocorticoids: anti-inflammatory and immunosuppressive actions

The most explored pharmacological effects of the glucocorticoids are the anti-inflammatory and the immunosuppressive ones.

Glucocorticoids impair several inflammatory mechanisms through the inhibition of inflammation mediators like for example prostaglandins and leukotrienes. The inhibition of the inflammation mediators is mainly achieved through the induction of

Annexin A1 (ANXA1) and MAKP phosphatase 1, and through the inhibition of NFKB and AP1 (Figure 5).

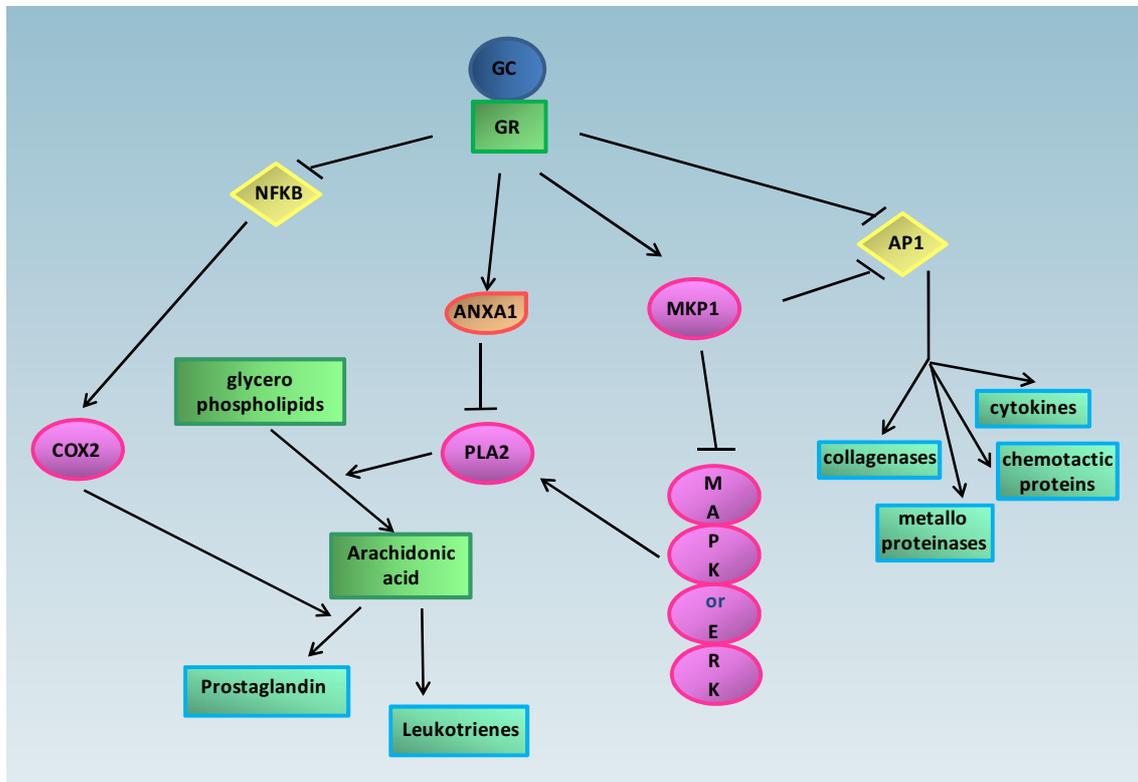


Figure 5. Major glucocorticoid targets involved in inflammation

ANXA1 can be induced by the glucocorticoids and it inhibits the synthesis of phospholipases A2 (PLA2).¹²¹ PLA2 hydrolyzes glycerophospholipids releasing arachidonic acid the precursor of the major inflammation mediators, prostaglandins and leukotrienes.

Glucocorticoids impair the activation of MAPK produced by inflammatory signals like virus, bacteria, and cytokines through the activation of MKP1. MKP1 desphosphorylates the activated MAPK proteins and consequently impairs MAPK cascades signaling.¹²² MAPK cascades are responsible for PLA2 activation. Thus, the activation of MKP1 by glucocorticoids inhibits the activation of PLA2 and the synthesis of the mediators of inflammation, prostaglandins, and leukotrienes. On the other hand, since MAPK cascades signaling activate JUN and the heterodimer JUN-FOS (AP1),

the activation of MKP1 also decreases the production of other mediators of inflammation that are regulated by AP1.

AP1 is a transcription factor that induces the expression of several inflammatory genes. Glucocorticoids can inhibit AP1 by direct interaction with the transcription factor, or indirectly, by inducing MKP1 that further inhibits AP1.¹²³ The inhibition of AP1 accounts for the anti-inflammatory action of the glucocorticoids since it impairs the production of the inflammation mediators regulated by AP1 like cytokines, chemotactic proteins, collagenases, and matrix metalloproteinases.

Glucocorticoids inhibit the transcription factor NF κ B through its retention in the cytoplasm, mimicking I κ B α inhibitory action.¹²⁴ Thus, glucocorticoids block the induction of the transcription of cyclooxygenase 2 (COX2) by NF κ B. COX2 is responsible for prostaglandin synthesis hence NF κ B inhibition accounts for the anti-inflammatory actions of glucocorticoids.¹²³ In addition, glucocorticoids were reported to interfere with other pro-inflammatory transcription factors such as IRF3, STAT, CREB, NFAT, TBX21, and GATA3.¹²⁵

Non-genomic effects in the regulation of inflammation have been described. For instance, in human endothelial cells the glucocorticoid-GR complex stimulates the activity of PI3K in a transcriptional independent manner. In turn, PI3K phosphorylates AKT, and AKT phosphorylates eNOS that once activated produces nitric oxide.¹¹³ Although nitric oxide is thought to be responsible for inflammation, mice experiments have shown that activation of the PI3K-AKT pathway by eNOS could have benefic repercussions.¹²⁶

A recent work has elucidated a novel non-genomic mechanism of action of glucocorticoids in T cells by its ligation to membrane-linked GR that further modulate signaling through TCR.¹²⁷ Glucocorticoids are able to abrogate the signaling through the TCR since they can dissociate the complex formed by TCR, LCK, and FYN.¹¹² The release of LCK and FYN suppress the phosphorylation of AKT, ERK, and other MAPK.

Many other effects on inflammation have been attributed to glucocorticoids like the inhibition of vasodilation, vascular permeability, and leukocyte migration. Moreover, glucocorticoids decrease the stability of the mRNA genes encoding pro-inflammatory proteins such as EGF and COX2.¹²⁸

The GR is expressed in virtually all cell types and thus glucocorticoid actions could be observed in immune cells (Figure 6).

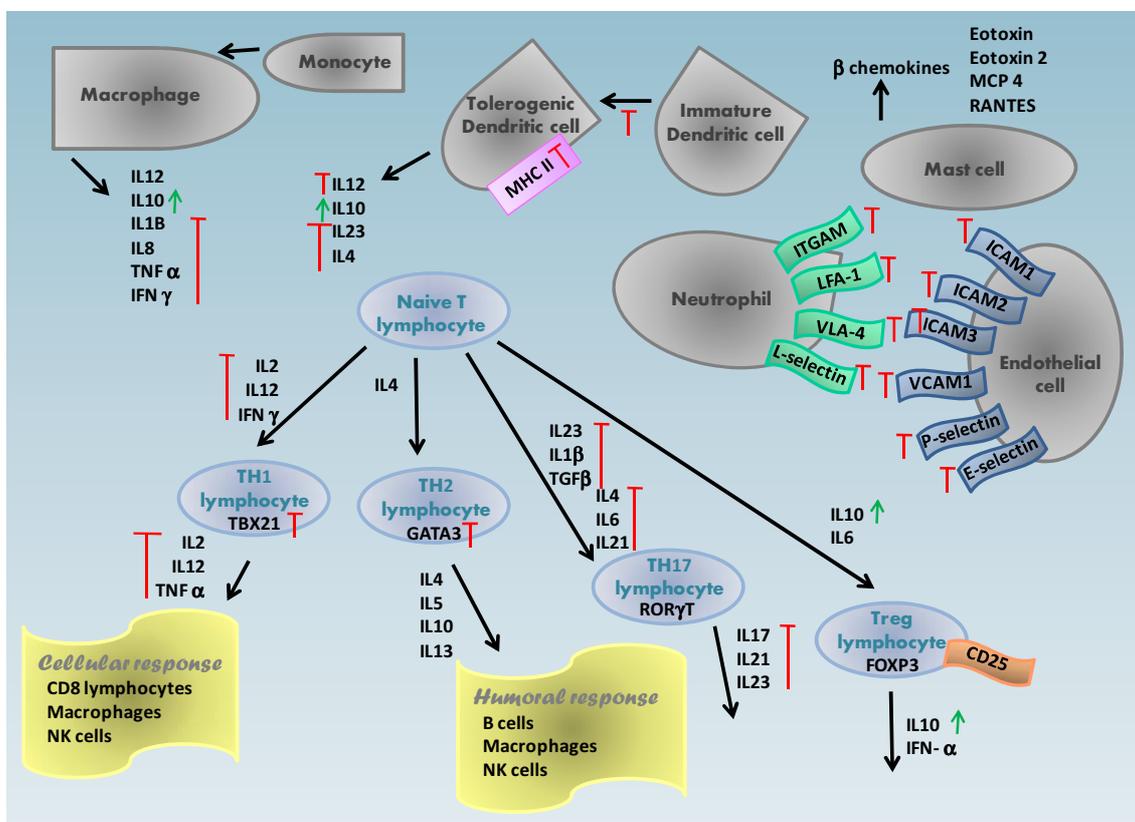


Figure 6. Glucocorticoids effects in the immune cells

Colour arrows are used to point glucocorticoid mediated actions, green arrows represent positive regulation by glucocorticoid, and red arrows represent negative regulation by glucocorticoids.

Glucocorticoids can modulate both arms of the immune system, the innate and the adaptive.¹²⁹ The innate immunity provides a non-specific response, and is the first

line of defense against invading pathogens. The adaptive immunity is the result of the production of high-affinity antibodies and thus is antigen specific and follows innate responses. Immune responses are performed by immune cells and some are components of the innate immunity like the antigen presenting cells (monocytes / macrophages, dendritic cells, and B cells), neutrophils, and NK cells. On the other hand, others like T cells are components of the adaptive immunity.¹²⁹

Dendritic cells are able to take antigens by endocytosis and present them through their MHCII receptors to antigen specific T helper cells. Glucocorticoids exert effects on dendritic cells on many levels of their life cycle. They arrest dendritic cell maturation and suppress dendritic cell activation by reducing the expression of MHCII, cytokines, and other co-stimulatory molecules. Importantly, glucocorticoids generate dendritic cells with tolerogenic properties, enhanced expression of IL10, and increased phagocytic activity.¹³⁰ Tolerogenic dendritic cells were shown to induce T cell anergy, T cell suppression, and the generation of regulatory T cells (Tregs). Moreover, they were shown to protect against autoimmune diseases and allograft rejection.¹³¹

Macrophages are important cells of the innate immunity; they recognize pathogens through membrane receptors as, for example, Toll-like receptors. After the ligation of the pathogens to those receptors, macrophages become activated and release a large repertoire of cytokines. Glucocorticoids efficiently suppress classical macrophage activation because they induce the synthesis of the immunomodulatory cytokine IL10, and because they inhibit the release of pro-inflammatory cytokines like $TNF\alpha$, $IFN\gamma$, and IL1B.¹³² Glucocorticoids activate GRE of genes like MKP1, and they interfere with transcription factors like NF κ B, AP1, and IRF3.¹³³ Interestingly, glucocorticoids induce an anti-inflammatory phenotype in macrophages. They increase macrophage phagocytic capacity by induction of protein S-dependent phagocytosis.¹³⁴ This accounts for a powerful anti-inflammatory action of glucocorticoids since macrophages eliminate the apoptotic neutrophils from the inflammation site.

Neutrophils are attracted to inflammation sites by chemokines released by mast cells, endothelial cells, and other myeloid cells. Rolling, adhesion, activation, and transmigration through the blood vessel are required steps for neutrophil homing to tissues, and glucocorticoids can affect all these steps. The interaction between neutrophils and endothelial cells is compromised by glucocorticoids since they decrease the expression of L-selectin, leukocyte integrins $\beta 1$ (VLA-4), and leukocyte integrins $\beta 2$ (LFA-1 and ITGAM) in neutrophils.¹³⁵ They also decrease the expression of these molecules counterparts, E-selectin, P-selectin, VCAM1, ICAM1, ICAM2, and ICAM3 in endothelial cells. On the other hand, glucocorticoids contribute to neutrophil survival and proliferation because they induce the expression of both proliferation receptors (GMCSFR, LTB4R) and survival molecules.¹³⁶ As a consequence, glucocorticoids increase the release of neutrophils from the BM, a finding that is exploited in order to overcome neutropenias. Notwithstanding, glucocorticoids are powerful anti-inflammatory compounds since they impair neutrophil migration to the inflammation sites.

Glucocorticoids have been used for a long time in the treatment of B cell related diseases; nevertheless, the mechanisms behind their actions have not been properly investigated. Initial reports on glucocorticoids chronic usage have shown that they reduce B cell numbers in spleen and lymph nodes, impair the differentiation of early B cell progenitors, decrease IgG production, and increase IgE.¹³⁷ Studies in pre B cell lines demonstrated that glucocorticoids impair the synthesis of BCL2, an anti-apoptotic protein over-expressed in some B cell malignancies.¹³⁸ Also, glucocorticoids can reduce the levels of BAFF, a member of the tumor necrosis factor family of proteins implicated in major steps of B cell development.¹³⁹ BAFF regulates lymphocyte survival and maturation, immunoglobulin production, immunoglobulin class switching, and stimulation of T cells. Taken together, the decrease in BCL2 and BAFF expression induced by glucocorticoids is able to induce apoptosis but only in specific B cell populations.

The effect of glucocorticoids in T cells has been extensively studied and different actions have been reported depending on the analyzed T cell subpopulations: immature, mature CD8+, and mature CD4+. For example, it was observed a gradation in the power of glucocorticoids to induce apoptosis; the immature T cell subpopulation CD4+CD8+ is very sensitive to apoptosis, the mature CD4+ subpopulation is quite sensitive, despite that the mature CD8+ subpopulation is only moderate sensitive.¹⁴⁰ The mechanism by which glucocorticoids induce apoptosis is mediated by an increase in the expression of the BH3 only pro-apoptotic proteins BIM and PUMA.¹⁴¹

Naïve CD4+ T cells are stimulated by antigens and can then differentiate into different subtypes: Th1, Th2, Th17, and Tregs. Each of these T helper subtypes expresses lineage specific transcription factors which are instructed by specific microenvironment cytokines combinations. Since glucocorticoids alter the expression of cytokines, they are able to affect differentiation of T helper cells.¹⁴⁰ Furthermore, the cytokines produced by one subtype of T helper cell inhibit the differentiation of other types of T helper cells.

Th1 cells are driven by IL2, IL12, and IFN γ , and express the TBX21 transcription factor. Through the activation of STAT4, Th1 cells produce and release pro-inflammatory cytokines such as IL2, IL12, IFN γ , and TNF α . Th1 cells are major players in the inflammatory process since their cytokines stimulate CD8+ effector T cells, NK cells, and macrophages. Subsequently, Th1 cells are promoters of cellular immunity. It is important to mention that Th1 cells are the predominant subtype of T cells in autoimmune diseases.¹⁴²

Th2 differentiation is induced by IL4. Th2 lymphocytes express the GATA3 transcription factor that further induces STAT6 function leading to the production of IL4, IL5, IL10, and IL13. Th2 cells effectively induce humoral immunity by stimulating B cells to produce antibodies and by activating mast cells and eosinophils.¹⁴²

The cytokine combination responsible for Th17 differentiation is not fully elucidated but includes IL6, IL23, IL21, IL1 β , and TGF β . Th17 cells express the ROR γ T

transcription factor which is able to activate STAT3 that further leads the production of IL17, IL21, and IL22.^{142;143} Th17 cells have been implicated in autoimmune diseases; elevated levels of IL17, which the major producers are Th17 lymphocytes, were found in PB and in tissues of patients with inflammatory bowel disease, psoriasis, and rheumatoid arthritis. Today is accepted that both Th1 and Th17 lymphocytes are independently capable of induce autoimmune diseases.¹⁴³

T regulatory cells (Tregs) derive from naïve Th0 lymphocytes and are characterized by the expression of CD4, CD25, CTLA4, and GITR, and of the transcription factor FOXP3. The differentiation of Tregs depends more on the signals received through the TCR than on the signals driven from the cytokine milieu. Although, it was observed that Tregs counts are increased by IL10 that is released from the tolerogenic dendritic cells, which points that cytokines are also important in the expansion of Tregs. Also TGF β and IL4 were shown to influence Tregs activity.¹⁴⁴ Tregs are able to impair effector T cell actions through a cell-to-cell contact mechanism and through the production of TGF β .¹⁴⁵

At physiological doses, glucocorticoids cause selective suppression of the Th1 cellular immunity axis and a shift toward Th2 mediated humoral immunity since they stimulate the production of IL4 and IL13, while they decrease the production of IL2, IL12, and IFN γ .¹⁴⁶ Notwithstanding, at pharmacological doses, glucocorticoids inhibit both Th1 and Th2 immune responses.¹⁴² Glucocorticoids suppress TBX21 action and impair STAT4 activity affecting Th1 differentiation. Moreover, they inhibit the nuclear import of GATA3 and suppress the STAT6 function interfering with Th2 differentiation. Glucocorticoids direct effects in Th17 differentiation have not been extensively studied but their effects on the cytokine milieu indicate that glucocorticoids are likely to impair Th17 differentiation. For instance, glucocorticoids decrease the production of IL23 by dendritic cells which is needed for Th17 differentiation.¹⁴⁷ Also, it was reported that glucocorticoids reduce IL6, IL17, and TGF β , supporting that glucocorticoids are able to

abolish Th17 differentiation.¹⁴⁸ Conversely, glucocorticoids are able to induce the generation of Tregs. The glucocorticoids were shown to increase Tregs counts by inducing the formation of tolerogenic dendritic cells.¹⁴⁹ All in all, the immunosuppression induced by glucocorticoids is achieved by impairing Th1, Th2, and Th17 responses, and by increasing Tregs responses.

In summary, the anti-inflammatory and the immunosuppressive effects of the glucocorticoids are due to their interference in several molecular mechanisms of different cell types. Because some of these mechanisms are also involved in physiological signaling, the therapeutic effects of glucocorticoids are often accompanied by clinically relevant side effects.

3. GLUCOCORTICOID USE IN LYMPHOID MALIGNANCIES

3.1. Effects of glucocorticoids on apoptotic cell death

The first observation of glucocorticoids apoptotic activity was disclosed when studying the physiological action of glucocorticoids in the control of T cell homeostasis. Glucocorticoids mediate the positive and negative selection of T cells in the thymus.¹⁴⁶ Then, it was observed that glucocorticoids induced apoptosis of leukemia, lymphoma and multiple myeloma (MM) cells, making them one of the most used drugs in the management of hematological malignancies.

Apoptosis is an encoded suicide program shared by the differentiated cells of multicellular organisms. Apoptosis regulates the elimination of cells that are no longer needed, have developed improperly, or have sustained genetic damage. Apoptosis is defined by a series of molecular and morphological events like chromatin condensation and fragmentation, cytoskeletal disruption, cell shrinkage, membrane blebbing, compaction of cytoplasmic organelles, dilation of the endoplasmic reticulum, and generation of apoptotic vesicles.¹⁵⁰ Apoptosis culminates in the orchestrated disassembly and in the phagocytosis of the dying cell. Lymphocytes can undergo two distinct apoptotic pathways, the intrinsic and the extrinsic.¹⁵¹ In addition, some reports have put in evidence alternative pathways like the destabilization of lysosomal membranes which is induced by lysosomal stress, and is accompanied with the release of Cathepsin B and D.¹⁵²

The intrinsic pathway is initiated by cellular stress or through the high affinity ligation of antigen receptors during the negative selection of T cells in the thymus. This pathway is regulated at the mitochondria level by BCL2 family members.¹⁵³ Briefly, cellular stress signals activate pro-apoptotic molecules of the BH3 only family like BIM, BID, BAD, BMF, PUMA, and NOXA that in turn activate the multidomain family members BAX and BAK. Of note, this could be neutralized by the anti-apoptotic BCL2

family members, BCL2 and BCLXL, which are able to abrogate the signaling of the pro-apoptotic molecules. Once activated, BAX and BAK migrate to the mitochondria membrane where they induce the formation of pores in the outer membrane and the consequent release of cytochrome c and SMAC / DIABLO.¹⁵⁴ Cytochrome c together with caspase 9 and APAF1 originate the apoptosome. This multimeric complex activates the effector caspase 3 that in turn cleaves the inhibitory subunit of DNases, activating their catalytic subunit that further fragments DNA. Caspase 3 also cleaves cytoskeletal proteins like foldrin and gelsolin, and induce the proteolysis of nuclear lamins, which in turn lead to cellular shape changes, nuclear shrinking, and budding. The release of SMAC / DIABLO in the cytoplasm allows its binding to IAPs like XIAP, cIAP1, or cIAP2 thus preventing the inhibition of caspase 3 and caspase 6 by these molecules.¹⁵⁵

The extrinsic pathway is initiated by the ligation of cell death receptors such as FAS (CD95).¹⁵⁶ The activation of these receptors subsequently activates caspase 8 that can directly activate the effector caspase 3. In some cell types, caspase 8 can also activate the pro-apoptotic BID leading to mitochondria destabilization and the initiation of cytochrome c mediated activation of caspase 3.¹⁵⁶

Glucocorticoids are able to induce apoptosis through interference with several apoptotic stimulus and mediators, and its action is diverse according to cell types (Figure 7).¹⁵⁵

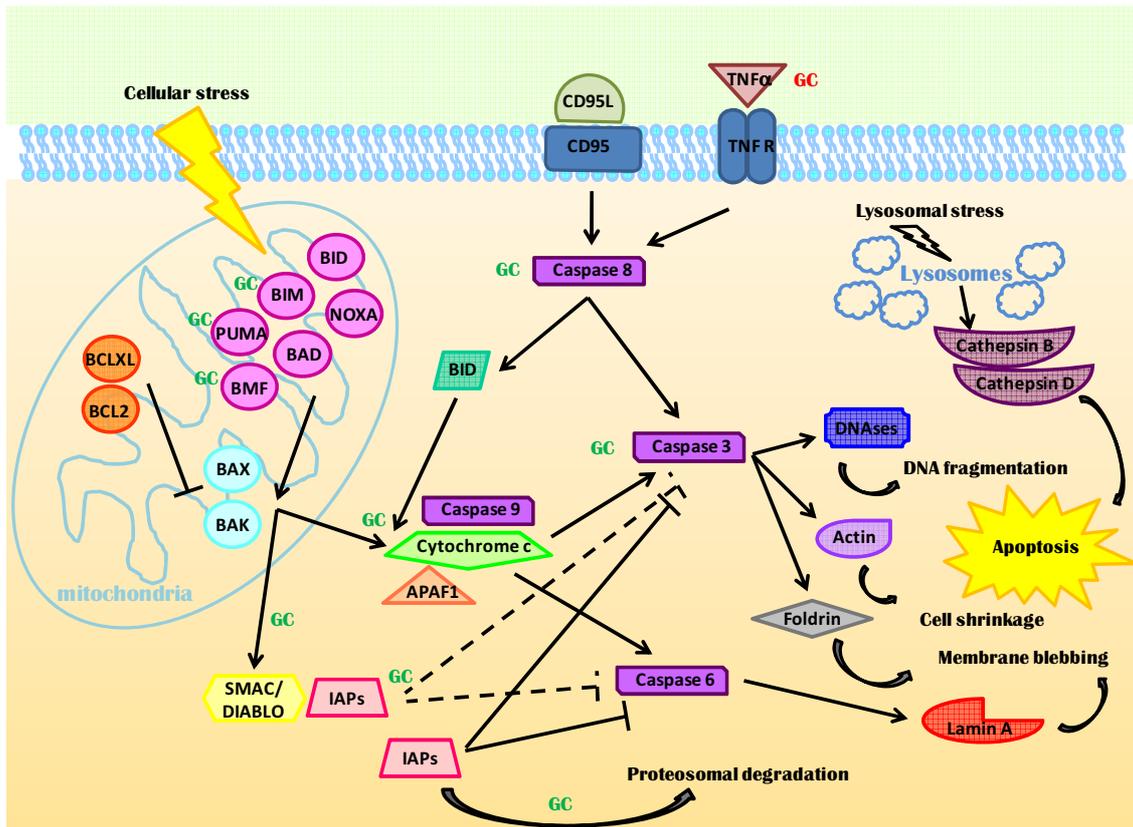


Figure 7. Glucocorticoids effects on apoptosis

Glucocorticoids actions in the intrinsic and extrinsic apoptotic pathways are marked with colored GC; green GC: positively regulated and red GC: negatively regulated.

Glucocorticoids can induce the intrinsic pathway. It has been reported that glucocorticoids induce the expression of the pro-apoptotic BIM, BMF and PUMA.¹⁵⁷ Of interest, the mechanism of apoptosis induced by the glucocorticoids through BIM is independent of TP53.¹⁵⁸ Cell treatment with glucocorticoid has been shown to lead to the activation of caspase 8, caspase 9, and effector caspase 3. Also glucocorticoids were shown to induce the degradation of XIAP and cIAP1 by the proteasome and thus to abrogate their inhibitory role in the activation of caspases. Because caspase 8 is an effector of the extrinsic pathway it cannot be ruled out that glucocorticoids activate the extrinsic apoptotic pathway. Nevertheless, evidence point to a minor role of extrinsic pathway in the apoptosis induced by glucocorticoids; first because glucocorticoids were

reported to impair the synthesis of $\text{TNF}\alpha$, a ligand of the TNF receptor, and second because glucocorticoids are able to induce apoptosis in the absence of BID.¹⁴⁰

Glucocorticoids require functional GR to mediate apoptotic events, since these often are due to genomic mechanisms like the transactivation of gene expression and gene transrepression.¹⁵⁹ However, some events may also involve non-genomic mechanisms.

Glucocorticoids are able to bind GRE of genes, and thus induce their transcription. While this mechanism (transactivation) may account for several of the glucocorticoids actions, especially those related with the regulation of metabolism, it probably plays a secondary role in the induction of apoptosis, since so far no GRE have been identified in pro-apoptotic genes. Many studies have been performed in order to identify genes regulated by the glucocorticoids with a role in apoptosis in lymphoid malignancies, especially in ALL.¹⁶⁰⁻¹⁶⁴ Nevertheless, when compared the results between different studies, few genes were commonly targeted by glucocorticoids, and many did not play a direct role in the apoptotic pathways. It is thought, that the apoptotic effects of the glucocorticoids rely on the targeting of multiple pathways, many of them involved in cell survival. Since, glucocorticoids inhibit the transcription of several pro-inflammatory and survival genes, this may account for cell apoptosis.¹⁵⁹

Glucocorticoids bind the transcription factor AP1 blocking its transactivation activity, thus resulting in the inhibition of the transcription of growth factors, cytokines, and survival genes.¹²³

Glucocorticoids impair the activity of the transcription factor NF κ B, an important mediator of cell survival, by several mechanisms.¹²³ Glucocorticoids induce the synthesis of the inhibitor of NF κ B, I κ B α , thus abrogating NF κ B translocation to the nucleus. Also, the glucocorticoids compete with NF κ B activators resulting in decreased NF κ B activity. Finally, the glucocorticoids have the ability to directly bind to NF κ B impairing its functions.

The transcription factor MYC regulates cell cycle and proliferation and it has been shown to be implicated in cell survival. Many leukemia and lymphoma cells show increased MYC expression, suggesting a role for MYC in the neoplastic transformation. The expression of MYC inhibits apoptosis and induces cell cycle arrest. In a variety of normal and malignant hematological cells, it has been reported that glucocorticoids are able to suppress MYC.¹⁵⁵ Moreover, it has been shown that the repression of MYC activity preceded the apoptosis induced by glucocorticoids.¹⁵⁵ MYC down-regulation may be directly involved in the initiation of apoptosis in leukemic cells. The mechanism by which glucocorticoids down-regulates MYC is still unknown.

Glucocorticoids induce GILZ expression by direct targeting since the promoter of GILZ possesses six GRE.¹⁶⁵ GILZ has been shown to possess anti-proliferative activity by negative regulation of RAS signaling.¹⁶⁶ GILZ associates with RAS and RAF impairing the phosphorylation of the downstream targets: ERK, AKT, and CCND1. GILZ was as well implicated in the inhibition of the transcription factors AP1 and NFkB.^{167;168}

It is important to mention that other events have been involved in the apoptosis induced by glucocorticoids like the production of hydrogen peroxide, the production of ceramide, the change in the intracellular levels of calcium and potassium, the inactivation of PI3K, and the induction of MKP1.¹⁵⁹ Of particular relevance, glucocorticoids have been shown to inhibit IL6 survival signaling.¹⁵⁹ Glucocorticoids not only impair IL6 production but they also activate RAFTK and repress signaling through STAT3, two molecules that are implicated in cell survival signaling mediated by IL6. Moreover, a crucial role for GSK3 has been reported in the transmission of the apoptotic signaling mediated by the glucocorticoids.¹⁶⁹ This kinase is associated with the GR in the absence of glucocorticoids being released upon binding. Once free, GSK3 interacts with BIM linking the GR with a pro-apoptotic effector.

3.2. Mechanisms of glucocorticoid resistance to apoptosis

Glucocorticoids are widely used in the treatment of lymphoid malignancies like ALL, NHL, MM, and CLL because of their ability to prevent the growth and to cause the apoptotic death of the malignant cells.¹⁷⁰ Glucocorticoids are included in the therapy protocols of ALL in combination with anthracyclines, vinca alkaloids, and asparaginase. Regimens composed by glucocorticoids, alkylating agents, anthracyclines, and vinca alkaloids are used in the management of NHL together with the anti-CD20 antibody in patients whose cells are CD20 positive. In MM, glucocorticoids are part of the front-line treatments: dexamethasone–vincristine-doxorubicin, prednisone-melphalan, dexamethasone-bortezomibe, dexamethasone-thalidomide, and dexamethasone-lenalidomid. In CLL, glucocorticoids are mainly included in second line therapeutic regimens and a particular interest is emerging on its use in 17p13 deleted cases.

In most of the patients, the treatment with glucocorticoids leads to a remarkable reduction of malignant cells. Notwithstanding, some tumors show primary resistance to glucocorticoids and others develop secondary resistance during treatment.¹⁷¹ The resistance to glucocorticoids can be absolute and irreversible, as for the case of non-functional GR, or it can be relative, translated in a decreased sensibility to the drug over time. In this case, it could be reverted by increasing the concentration of the glucocorticoid. Multiple mechanisms could lead to glucocorticoid resistance and they could be grouped in upstream and downstream mechanisms. The former implicates the glucocorticoid receptor and co-chaperone proteins, they are often associated with primary and absolute resistance; the latter are the most common and normally are acquired during treatment, and they are the result of defects in components of the glucocorticoid pathway, or of cross-talk from other signaling pathways that interfere with the glucocorticoid one.

3.2.1. Upstream mechanisms of glucocorticoid resistance

The upstream mechanisms of glucocorticoid resistance include: pre-receptor defects, impaired GR expression, and deficiencies in co-chaperone proteins of the GR. The term pre-receptor defect is applied to features that reduce the levels of the available glucocorticoid. The MDR1 gene encodes for P-glycoprotein 1, a transporter protein that pumps lipophilic drugs out of the cell. The MDR1 gene is frequently over-expressed in malignant cells and is responsible for glucocorticoids and other drugs resistance, since it impairs the concentration of lipophilic drugs within the cell.¹⁷² Another mechanism that reduces the levels of glucocorticoids is their inactivation by enzymes such as 11 β -hydroxysteroid dehydrogenase. A recent report has shown that high levels of 11 β -hydroxysteroid dehydrogenase are associated with the resistance of T-lymphoblastic leukemia cells to prednisolone.¹⁷³

The impaired expression of functional GR can result from insufficient GR expression, from loss of function of GR due to mutations, and from expression of GR variants with reduced activity. Early studies with ALL cell lines resistant to glucocorticoid induced apoptosis have allowed the identification of numerous mutations in the GR gene that lead to its loss of function. Nevertheless, mutations in the GR are rarely found in patients with primary or relapsed ALL.¹⁷⁴ No evidence of GR mutations in patients treated with combined chemotherapy has been reported, despite it is known that chemotherapeutic regimens are likely to induce gene mutations.¹⁷⁵ Intensive research has been made in order to ascertain the functionality of the different variants of the GR and their implications in the resistance to apoptosis, but no consensus has been reached so far. The major functional variant is the GR α , the other variants lack or present shorter transactivation and ligand binding domains. Both transactivation and alterations in ligand domains account for impaired GR activity. For example, the GR β lack transactivation activity and a deficient ligand binding domain, and it has been

implicated in resistance to glucocorticoids in lymphoblastic cell lines.¹⁷⁶ Conversely, other reports haven't found a correlation between the levels of GR β and the resistance to glucocorticoids.^{177;178} Importantly, the basal levels of GR α present in the cell as well as the auto-induction of the GR seem to be critical to the sensitivity to apoptosis induced by glucocorticoids. Although this remains to be elucidated, since in some models glucocorticoids do not up-regulate the GR.^{109;179}

The GR is present in the cytoplasm associated with co-chaperone proteins that regulate its proper folding, the binding to glucocorticoids, and subsequent nuclear translocation. Furthermore, in the nucleus, the GR may recruit co-factors necessary for its gene regulatory activities. The levels of the GR co-chaperone proteins have been studied in ALL primary cells and no relationship has been found between the levels of HSP70, HSP90, HSP40, HIP, HOP, FKBP5, FKBP4, PPID, BAG1, and P23, and the resistance or sensitivity to glucocorticoids.¹⁸⁰ Nevertheless, other studies have described that BAG1, HSP90, and HSP70 expression levels affected the ability of glucocorticoids to induce apoptosis.^{171;181;182}

3.2.2. Downstream mechanism of glucocorticoid resistance

Glucocorticoids induce apoptosis by interference with multiple signaling networks, and resistance can come from deregulated activity of any of the components of those networks. Importantly, resistance could result from over-expression of anti-apoptotic proteins or from increased signaling through survival pathways that counteract the apoptotic actions of the glucocorticoids.¹⁷¹

The expression of anti-apoptotic proteins is a frequent feature of leukemic cells and has been associated with resistance to glucocorticoids. For instance, over-expression of BCL2 in ALL cell lines has been shown to confer resistance to apoptosis.¹⁸³ BCLXL was suggested to play a role in the protection of leukemic cells to

undergo apoptosis. Also, BCLXL was thought to predict responses to glucocorticoid treatment of ALL patients.¹⁸⁴ Moreover, increased expression of MCL1 is frequently observed in the gene expression signature of glucocorticoid resistant cells.¹⁶² Although the status of the BCL2 rheostat influences the sensitivity to glucocorticoid induced apoptosis, the expression of the pro and anti-apoptotic BCL2 family members is altered during the glucocorticoid exposure, and some of the protective effects of the anti-apoptotic proteins could be reverted during long term treatments.¹⁷¹ In summary, the over-expression of anti-apoptotic proteins could influence the response to glucocorticoids, but the net result depends on the cellular context and of additional signals feeding into the BCL2 rheostat.¹⁵⁷

Glucocorticoids impair the signaling of several survival pathways like the ones mediated by PI3K / AKT / mTOR, RAS / RAF / MEK / ERK, and JAK / STAT. The increased activation of such pathways has been related to resistance to glucocorticoid induced apoptosis. The loss of PTEN, a negatively regulator of PI3K / AKT signaling, is a common venue of T ALL, and the hyperactivation of the AKT pathway is frequently observed.^{185;186} AKT prevents apoptosis by impairing the activity of BAD, caspase 9, and GSK3, and by increasing the activity of IKK and MDM2.¹⁸⁷ Importantly, AKT has been shown to antagonize the apoptosis induced by the glucocorticoids in T ALL, T cell lymphoma, and follicular lymphoma cells.^{169;188} The importance of mTOR in the resistance to glucocorticoids is disclosed in the finding that mTOR inhibitors like rapamycin are able to sensitize MM, T ALL, B ALL, and Burkitt lymphoma cells to glucocorticoid induced apoptosis.¹⁸⁹

The RAS / RAF / MEK / ERK survival pathway counteracts apoptosis induced by glucocorticoids. In glucocorticoid resistant cell lines from T ALL, MM, T cell lymphoma, and Burkitt lymphoma, inhibition of ERK renders the cells sensitive to apoptosis; the same was observed in ALL primary cells.¹⁸⁹ The treatment of ALL cells with inhibitors of MEK / ERK results in increased expression of BIM and activation of BAX.¹⁹⁰ The JAK / STAT pathway is activated by the ligation of IL6 to its membrane

receptor. In MM, the autocrine production of IL6 is correlated with a highly malignant phenotype and with resistance to dexamethasone induced apoptosis.¹⁹¹ Moreover, other components of this pathway, for instance STAT3, were shown to be constitutively activated in some hematological malignancies, whereas the inhibition of STAT3 had rendered the cells sensitive to apoptosis.^{192;193}

3.3. Glucocorticoids in the therapeutic management of CLL

Treatment of patients with CLL has dramatically changed during the last decade with the introduction of monoclonal antibodies. Chemoimmunotherapy regimens like FCR (fludarabine, cyclophosphamide and rituximab),^{69;194} FCR plus mitoxantrone,^{195;196} or FCR plus alemtuzumab,¹⁹⁷ have proved to be highly effective in the treatment of this disease. Despite the excellent overall response and complete response rates obtained with these regimens, patients with 17p13 deletion and / or TP53 mutations usually exhibit a lower response rate, shorter progression-free survival, and overall survival.^{8;69;198} Moreover, there are patients for whom purine analog-base therapies are inappropriate, namely for those suffering from renal dysfunction due to the fact that purine analogs are eliminated predominantly through the kidneys.

The activity of glucocorticoids on CLL cells and in patients with CLL has been reported for many years. In the early nineties, it was unveiled that glucocorticoids induce the death of CLL cells by apoptosis,¹⁹⁹ they were shown to induce DNA fragmentation.²⁰⁰ This process was mediated by caspases that were able to cleave PARP, a group of enzymes involved in DNA repair.²⁰¹ Afterwards, it was shown that the conformational changes induced by the glucocorticoids in BAX and BAK were associated with the induction of apoptosis. Importantly, these changes preceded the activation of caspases and were independent of p53.²⁰² By that time, it was already known that CLL cells show considerable variability in the sensitivity to glucocorticoids, yet neither the basal levels of BAX nor the levels of the anti-apoptotic protein BCL2 were found to be related.²⁰³ Furthermore, it has been reported that glucocorticoids up-regulate mRNA and protein expression of the pro-apoptotic BIM.²⁰⁴ This finding supported the involvement of the BCL2 rheostat in the apoptosis induced by the glucocorticoids and was in line with previous observations reporting the independence of p53.

Additional insights in the apoptotic mechanisms of action of the glucocorticoids were provided by synergistic studies of glucocorticoids with inhibitors of the proteasome, of phosphodiesterase 4, of BCL2, and of LCK.²⁰⁴⁻²⁰⁸ The combined use of glucocorticoids and BCL2 inhibitors led to an increase in apoptosis which underscored the role of the BCL2 rheostat in the induction of the apoptosis by the glucocorticoids.²⁰⁷ Furthermore, it has been observed that survival signals that activated AKT and ERK induced the phosphorylation and further degradation of BIM by the proteasome.²⁰⁴ For this reason, it was suggested that proteasome inhibitors were able to increase the apoptosis induced by the glucocorticoids through an increase in BIM levels. Importantly, a link between the levels of BIM and the activation of AKT and ERK pathways has been reported, pointing out that the survival signals mediated by those pathways are behind the sensitivity to glucocorticoids. A recent work correlated positively the levels of LCK with the response to glucocorticoids, and showed that inhibition of LCK synergizes with glucocorticoids.²⁰⁸ LCK regulates the BCR activity, and LCK is aberrantly expressed in CLL cells. All in all, the results of this study are indicative that signaling through BCR can affect the response to glucocorticoids, and that the impairment of survival signals mediated by the BCR may sensitize cells to apoptosis.

Of major interest was the finding that cell death induced by glucocorticoids is higher in CLL with unmutated IGHV genes / high ZAP70 expression than in cases with mutated IGHV genes / low ZAP70.²⁰⁹⁻²¹² Boelens *et al* explored the possible influence of ZAP70 expression in the different responses to glucocorticoids.²¹¹ They found that glucocorticoids decreased the expression of ZAP70 and SYK, a positive effector of the responses mediated by the BCR. They also observed that glucocorticoids induced the expression of PTP1B, an enzyme that dephosphorylates SYK. The inhibition of PTP1B restored the expression of ZAP70 and the phosphorylation of SYK, but it did not affect the response to glucocorticoids. The levels of ZAP70 and the activity of SYK *per se* were not responsible for different glucocorticoid sensitivity.

From the therapeutic standpoint, glucocorticoids are a feasible therapeutic option for patients with refractory disease, particularly those with TP53 abnormalities. In some clinical studies glucocorticoids were used alone, and were administered to previously treated patients, most of them with TP53 abnormalities.²¹³⁻²¹⁶ More recent publications strengthen the benefit of the combination of glucocorticoids with monoclonal antibodies such as anti-CD20 and anti-CD52.²¹⁷⁻²²¹ Finally, because of their immunosuppressive properties, glucocorticoids are indicated for the management of autoimmune diseases associated with CLL like autoimmune hemolytic anemia, idiopathic thrombocytopenia, and pure red cell aplasia.

Preliminary data in a short series of patients with CLL obtained before the beginning of this project were in line with previous reports: the CLL cases with unmutated IGHV genes / high ZAP70 expression had better responses to dexamethasone than the cases with mutated IGHV genes / low ZAP70 expression.²⁰⁹⁻²¹² This finding prompted the study of the different effects of glucocorticoids in the CLL groups defined by the mutation load of the IGHV genes and the expression of ZAP70. Moreover, with the increasing use of glucocorticoids in refractory and TP53 deleted / mutated CLL cases, the understanding of the differential effects of glucocorticoids in patients with CLL gained further interest. In this line, the disclosing of the molecular mechanism responsible for different drug sensitivities could allow the identification of particular groups of patients prone to benefit from glucocorticoid based therapies.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Glucocorticoids are frequently included in the chemotherapy regimens administered to patients with CLL because they are potent immunosuppressant agents and because they are able to induce apoptosis in CLL cells. Although used from a long time, the molecular mechanisms by which glucocorticoids induce cell death in CLL cells are largely unknown. Interestingly, CLL cells from prognostic groups defined by the mutational load of the IGHV genes and the expression of ZAP70 seem to have different responses to glucocorticoids.

The hypothesis in this thesis is that in CLL, there are genes or proteins that determine the different response to glucocorticoids among the specific prognostic groups of patients. The identification of those genes would contribute to the general knowledge of the CLL biology and would direct the design of glucocorticoid based therapies to particular groups of patients.

OBJECTIVES

1. To explore the differential response to dexamethasone in different groups of CLL, defined by the mutational load of the IGHV genes and /or ZAP70 expression.
2. To analyze the role of BIM in the apoptosis of CLL cells induced by dexamethasone.
3. To study the molecular mechanisms regulated by dexamethasone responsible for the apoptosis of CLL cells in groups defined by the mutational load of the IGHV genes / ZAP70 expression.

MATERIALS AND METHODS

1. PATIENTS SELECTION AND SAMPLE COLLECTION

A group of 50 patients from our institution with CLL diagnosis was selected on the basis of the availability of frozen samples for biological studies. Informed consent from all patients was obtained according to the Declaration of Helsinki, and the study was approved by the ethic clinical research committee of the Hospital Clinic Barcelona, Spain. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, United Kingdom). For that, heparinized peripheral blood was diluted with equal volume of phosphate buffered saline solution (PBS buffer) 1x (Roche Diagnostics GmbH, Mannheim, Germany) and transferred to a Falcon tube with Ficoll (half of the diluted blood volume). Then, it was centrifuged at 2000 rpm for 20 minutes at room temperature (RT). The PBMC fraction was retrieved for other tube, and washed twice with PBS buffer 1x by centrifugation at 1500 rpm for 5 minutes. Finally, cells were resuspended in fetal bovine serum (FBS) (Gibco, Paisley, Scotland, UK) in a concentration of 20×10^6 cells/ml.

CLL PBMC were frozen in DMSO (Sigma-Aldrich, Madrid, Spain) and stored at -180°C until analysis. Cryopreservation media consisted in a mixture of three parts of DMSO, one part of FBS, and one part of RPMI medium (Gibco). The same volume of cryopreservation media was added gently to the PBMC cells resuspended in FBS. Cells were immediately frozen at -80°C and further stored at -180°C .

2. CULTURE OF CLL CELLS

CLL PBMC were thawed at 37°C, placed in RPMI with 10% FBS, and immediately centrifuged at 1500 rpm for 10 minutes. Then, cell pellet was resuspended in culture media in a final concentration of 1×10^6 cells/ml. PBMCs were allowed to recuperate from thawed one hour in incubator before any manipulation. After this period, cell viability was accessed by surface annexin V binding and propidium iodide (PI) staining flow cytometry analysis as described in continuation (point 3 of this section).

3. DETERMINATION OF CELL VIABILITY

Cell viability was determined by flow cytometry by means of surface annexin V binding and propidium iodide (PI) staining. Annexin V binds phosphatidylserine residues, and PI binds nucleic acids. Phosphatidylserine is normally present in the intracellular layer of the cytoplasmic membrane of mammalian cells. Due to cell membrane reorganization during apoptosis, phosphatidylserine moves to the extracellular layer. Its detection on the extracellular layer is used as a marker of early-apoptosis. On the other hand, PI cannot pass the cell membrane and it is generally excluded from viable cells. Only cells with a damaged cell membrane allowed the entry and further detection of PI. Thus, PI stains late-apoptotic or necrotic cells.

According to manufacturer procedure (rh Annexin V/FITC kit, Bender MedSystems, Vienna, Austria), 2.5×10^5 to 5×10^5 cells were placed in PBS buffer 1x and centrifuged at 2000 rpm for 5 minutes at RT. Supernatant was removed and 200 μ l of cool annexin buffer were added, and cells were resuspended. Then, 0.5 μ l of annexin V labeled with fluorescein isothiocyanate (FITC) were added to cell suspension, and were incubated for 5 minutes at RT. After that, 2 μ l of PI were added, and cells were immediately acquired on a FACScanTM cytometer (Becton and Dickinson, Qume Drive, San Jose, CA) using the CELLQuestTM software (Becton and Dickinson). Analyses were made with the Paint-A-GateTM software (Becton and Dickinson) taken in consideration that: early apoptotic cells stain solely for annexin V, late apoptotic cells stain for PI and annexin V, necrotic cells stain only for PI, and live cells are negative for all the stains employed. As showed in Figure 8 cell viability was determined in the lymphocyte gate and was given as the percentage of live cells in the gate.

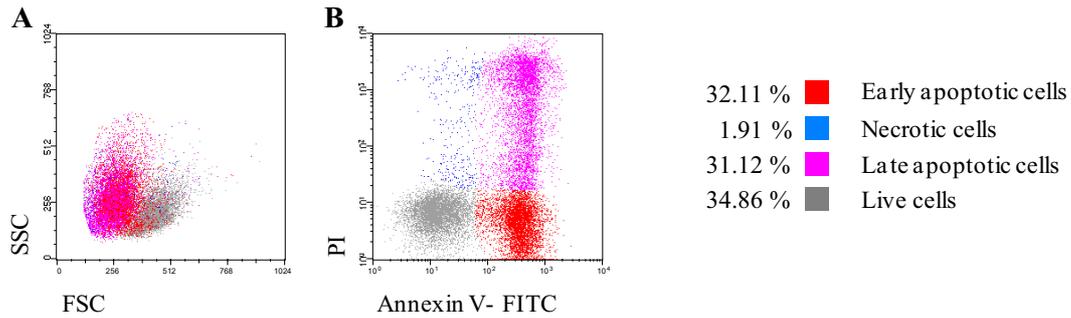


Figure 8. Cell viability determination by flow cytometry by means of surface annexin V binding and propidium iodide (PI) staining

(A) Lymphocytes were gated according to FSC/SSC characteristics. (B) In the lymphocytes gate, cells were further analyzed for annexin V and PI staining. Early apoptotic cells only stain for annexin V, red events. Necrotic cells only stain for PI, blue events. Late apoptotic cells stain for annexin V and for PI, purple events. Live cells do not stain for any of the employed dye, grey events.

The viability of the cells was determined after the recover from thawing in culture for 1 hour. Only CLL samples with more than 50% of live cells were further used.

4. TREATMENT OF CLL CELLS WITH DEXAMETHASONE AND EVALUATION OF RESPONSE

CLL cells were treated *ex-vivo* with the glucocorticoid dexamethasone (DXM; Merck Farma y Quimica SL, Mollet del Valles, Spain) at a concentration of 13.25 μ M based on previous reports.²⁰¹ For that, CLL cells were split in two, for control, and for incubation with DXM. After 24 hours, cell viability of both treated, and control cells, was evaluated by flow cytometry by means of surface annexin V binding and PI staining. The response to DXM was calculated as the percentage of live cells after treatment with DXM relative to the percentage of live cells in the untreated cells (left with standard medium):

$$\text{Response to DXM (\%)} = \frac{\% \text{ of live cells in culture with DXM} \times 100}{\% \text{ of live cells in untreated culture}}$$

5. PROTEIN ANALYSIS

5.1. Protein analysis by flow cytometry

The protein analysis by flow cytometry was performed in 5×10^5 cells previously labeled. For that, 5×10^5 cells were retrieved from the culture and washed with PBS buffer 1x by centrifugation at 1500 rpm for 5 minutes at RT. Then, they were resuspended in approximately 100 μ l of PBS buffer 1x for further membrane or intracellular protein staining. The antibodies used are listed in Table 3.

Table 3. Antibodies used in flow cytometry determinations

Antibody	Source	Clone	Isotype	Fluorochrome	Supplier
anti-CD3	mouse	SK7	IgG + IgK	PE	Becton and Dickinson, San Jose, CA
anti-CD56	mouse	MY31	IgG + IgK	PE	Becton and Dickinson, San Jose, CA
anti-CD19	mouse	SJ25C1	IgG + IgK	PerCP-Cy5.5	Becton and Dickinson, San Jose, CA
anti-CD5	mouse	UCHT2	IgG + IgK	APC	BD Biosciences Pharmingen, San Diego, CA
anti-ZAP70	mouse	2F3.2	IgG2a		Upstate, Lake Placid, NY
anti-mouse	goat		F(ab') ₂	FITC	Dako, Glostrup, Denmark

5.1.1. Infiltration of tumor cells

The CLL cells express the pan B receptor CD19 and CD5 on opposite to normal mature B cells. Thus, CLL cells were identified by the concomitant expression of CD19 and CD5. 10 μ l of anti-CD19 labeled with peridinin chlorophyll protein-cyanin

5.5 (PerCP-CyTM5.5) and 5 ul of anti-CD5 labelled with allophycocyanin (APC) were added to 5×10^5 cells previously washed and resuspended in PBS buffer 1x. After incubation for 15 minutes at RT, the cells were washed with PBS buffer 1x and centrifugated at 1500 rpm for 5 minutes at RT. Then, they were immediately acquired in a BD FACSCaliburTM cytometer (Becton and Dickinson) using the CELLQuestTM software. Analyses were made with the Paint-A-GateTM software; lymphocytes were gated based on their forward scatter / side scatter (FSC / SSC) characteristics, and the percentage of CLL cells was determined as the percentage of double positive cells for CD19 and CD5 in the lymphocyte gate.

5.1.2. Analysis of ZAP70 expression

ZAP70 is an intracellular protein and for this reason, cells had to be fixed and permeabilized before staining. To 5×10^5 cells previously washed and resuspended in 100 ul of PBS buffer 1x, equal volume of solution A (Fix and Perm, Caltag Laboratories, Paisley, UK) were added, and the cells were incubated for 15 minutes at RT. Cells were subsequently washed with PBS buffer 1x at 1500 rpm for 5 minutes and resuspended in 100 ul of PBS buffer 1x. Then, equal volume of solution B (Fix and Perm) and 1.5 ug of anti-ZAP70 were added, and cells were incubated for 20 minutes at RT. Cells were washed twice with PBS buffer 1x at 1500 rpm for 5 minutes. Subsequently, 1 ul of goat anti-mouse immunoglobulin FITC was added, and cells were incubated for 20 minutes at dark at RT. Cells were washed in PBS buffer 1x at 1500 rpm for 5 minutes and were incubated for 5 minutes with 5 ul normal mouse serum (Dako, Glostrup, Denmark) at RT. After this, the following antibodies were added: 10 ul of anti-CD3 phycoerythrin (PE), 10 ul of anti-CD56 PE, 10 ul of anti-CD19 PerCP-Cy5.5, and 5 ul of CD5 APC. Cells were allowed to incubate 15 minutes at dark at RT, were washed in PBS buffer 1x at 1500 rpm for 5 minutes, and were acquired in

a BD FACSCalibur™ cytometer. At least 1000 cells CD56 / CD3 positive were acquired per sample, and both acquisitions and analyses were done with the CELLQuest™ software.

The expression of ZAP70 in CLL cells was calculated as the percentage of positive cells, using the expression of ZAP70 in sample T lymphocytes and NK cells as internal positivity control.⁵⁷ Lymphocytes were gated according to their FSC / SSC characteristics. Further, T lymphocytes and NK cells (CD3 and CD56 positive cells) were gated, as well as CLL cells (CD19 and CD5 positive cells). Biparametric dot graphs were constructed for T and NK cells, and for CLL cells. In the T and NK cells graph, two populations were separated according to ZAP70 expression. The cut-off value that separate ZAP70 positive from ZAP70 negative cells in the former graph, was applied in the graph of the CLL cells allowing the identification of the CLL cells positive for ZAP70. ZAP70 expression was given in percentage of total CLL cells. CLL cases were considered to be positive for ZAP70 expression when the percentage of ZAP70 positive cells was above 20%.⁵⁷ More details of this analysis are provided in Figure 9.

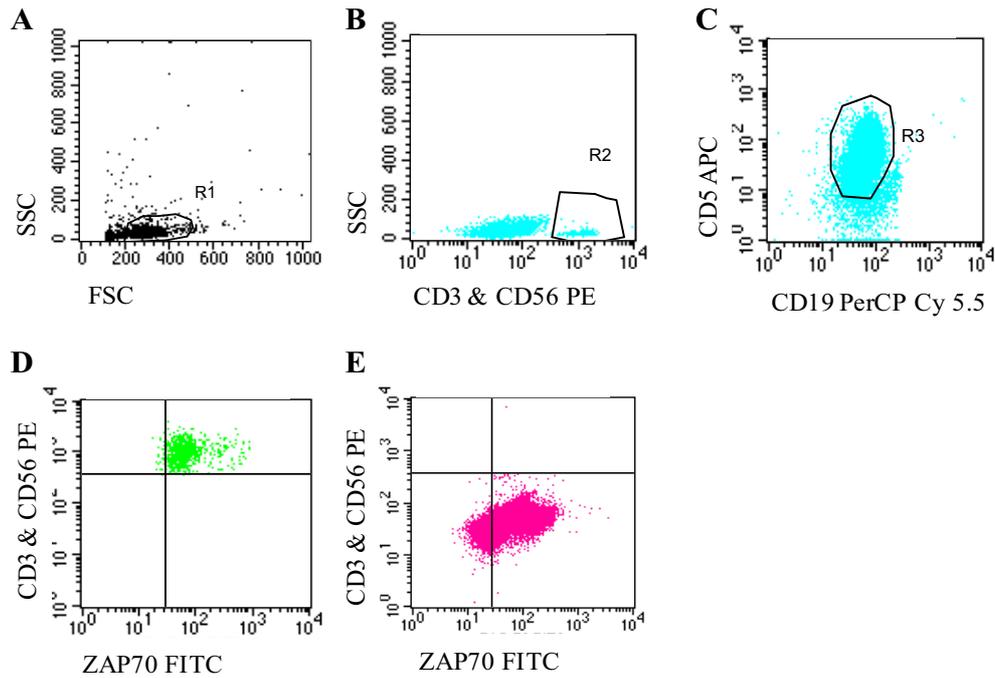


Figure 9. Determination of ZAP70 expression by flow cytometry

(A) Lymphocytes were gated (R1) according to FSC / SSC characteristics. (B) R1 events were analyzed for CD3 and CD56 expression and positive cells (T and NK cells) were gated (R2). (C) R1 events were studied for CD19 and CD5 expression, the positive cells for both markers (CLL cells) were gated (R3). (D) T and NK cells (R2 and R1 events) were analyzed for ZAP70 expression, quadrant axis were defined based on the fact that both T and NK cells are positive for CD3, CD56, and ZAP70. (E) CLL cells (R3 and R1, and not R2) were analyzed for ZAP70 expression using the quadrant axis defined in D.

5.2. Protein analysis by immunoblotting

5.2.1. Preparation of total protein cell lysates

Total protein cell lysates were prepared from CLL samples. For that, 100 ul of lysis buffer (20 mM Tris pH 7.4, 1 mM EDTA, 140 mM NaCl, and 1% NP-40) supplemented with 1x proteases inhibitor cocktail (BD Baculo Gold™, BD Bioscience Pharmingen, San Diego, CA) and 2 mM Na₃VO₄ were added to the pellet of 5x10⁶ cells, and were incubated for 30 minutes on ice. Then, the suspension was centrifuged at 14000 rpm for 2 minutes at RT, and supernatant (cell lysate) was recovered to another eppendorf.

5.2.2. Total protein cell lysates quantification: Bradford method

Protein quantification was based on the Bradford method by means of the Bio-Rad Protein Assay (Bio-Rad Laboratories, München, Germany). The dye reagent concentrate was diluted at 40% in distilled water. A stock solution of albumin at 0.1 mg/ml in water was prepared, furthermore, five standard dilutions of albumin (1, 2.5, 5, 7.5, and 10 ug/ml) were prepared. Then, 2 ul of lysis buffer were added to 488 ul of each standard dilution, and 2 ul of each total protein cell lysate were added to 488 ul of water. All solutions were mixed with equal volume (500 ul) of diluted dye reagent, and were incubated for 10 minutes. The absorbance of the solutions was measured at 595 nm.

The values of the concentration and the absorbance of the standards were used to construct a graph, and the linear regression curve obtained was used to extrapolate the value of the protein concentration of cell lysates.

5.2.3. Protein separation and blotting

Proteins were first separated by gel electrophoresis based on their physical properties and then transferred to a synthetic membrane. Accordingly, 25 ug of whole cell proteins were separated on a 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and were transferred to an Immobilon-P membrane (Millipore, Bedford, MA).

The membranes were blocked for 1 hour at RT with TBST buffer solution (20 mM Tris pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% of non fat dry milk (blocking buffer solution). The membranes were ready to be incubated with primary antibodies.

5.2.4. Immunostaining and analysis of FKBP5 expression

The FKBP5 protein has 51 kDa and GAPDH, the loading control, has 36 kDa. Hence, previous to the incubation with the corresponding primary antibody, the membranes were cut in two halves at the level of the 40 kDa proteins. The upper halves contained the higher mass proteins and were incubated over night at 4°C with the anti-FKBP5 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) diluted at 1:500 in blocking buffer solution. The lower halves were incubated over night at 4°C with anti-GADPH antibody (Abcam, Cambridge, UK) diluted at 1:1000 in blocking buffer solution.

After extensive wash with TBST buffer solution, the detection of the proteins was performed using peroxidase linked antibodies that further catalyzed a chemiluminescent reaction. The upper and the lower halves of the membranes were incubated 1 hour respectively with anti-goat IgG Horseradish Peroxidase secondary antibody (Dako, Glostrup, Denmark), and with anti-rabbit IgG Horseradish Peroxidase

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secondary antibody (Dako, Glostrup, Denmark), in blocking buffer solution. Afterwards, the membranes were washed twice with TBST, and a third wash with TSB buffer solution (20 mM Tris pH 7.5, and 150 mM NaCl) was made. The chemiluminescent substrate ECLTM Western blotting detection system (Amersham Bioscience, Buckinghamshire, UK) was added. Images were captured with LAS-3000 imaging system (Fuji Photo Film Co., Carrolton, TX) and analyzed using the Image Gauge V4.0 software (Fuji Photo Film Co.). The expression of FKBP5 was normalized to the expression of GAPDH, and was expressed in arbitrary units (AU).

6. RNA EXTRACTION, QUANTIFICATION AND QUALITY CONTROL

RNA was extracted from 5×10^6 cells with Trizol reagent (Invitrogen Life Technologies, Paisley, Scotland, UK). The cells were washed with PBS 1x, and 1 ml of Trizol reagent was added to the pellet. Cells were disrupted and homogenized with a syringe and needle. Then, 200 μ l of chloroform (Sigma-Aldrich Inc, St Louis, MO) were mixed vigorously with the homogenate. Two phases were distinctly separated after centrifugation at 11,400 rpm for 10 minutes at 4°C. The aqueous phase contained the RNA, and was collected to another eppendorf. The RNA was precipitated over night at -20°C with equal volume of 2-propanol (Sigma-Aldrich Inc, St Louis, MO), and was retrieved by centrifugation at 14,000 rpm for 10 minutes at 4°C. Afterwards, RNA was washed twice with 1 ml ethanol 75% (prepared by dilution of absolute ethanol (Merck, Darmstadt, Germany) in DEPC H₂O (Ambion, Foster City, CA)), and was dissolved in DEPC water. The RNA was immediately used, or stored at -80°C.

The quantification of the RNA was made in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA) at 260 nm. The quality of the RNA was assessed with an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA) (Figure 10). Briefly, the RNA samples were separated by electrophoresis in microfabricated chips, and the RNA fragments were visualized via laser induced fluorescence detection. Then, the software generated an electropherogram and a gel-like image that allowed the visualization of the integrity of the RNA samples. Moreover, the software calculated the ribosomal ratio (ratio between the ribosomal subunits 28S and 18S) and the RIN (RNA integrity number). The RIN algorithm attributed RNA samples a number from 1 to 10 to score their integrity, being 1 the most degraded and 10 the most intact.

RNA quality is of major importance in microarray analysis and only samples with RIN above 8 were processed.

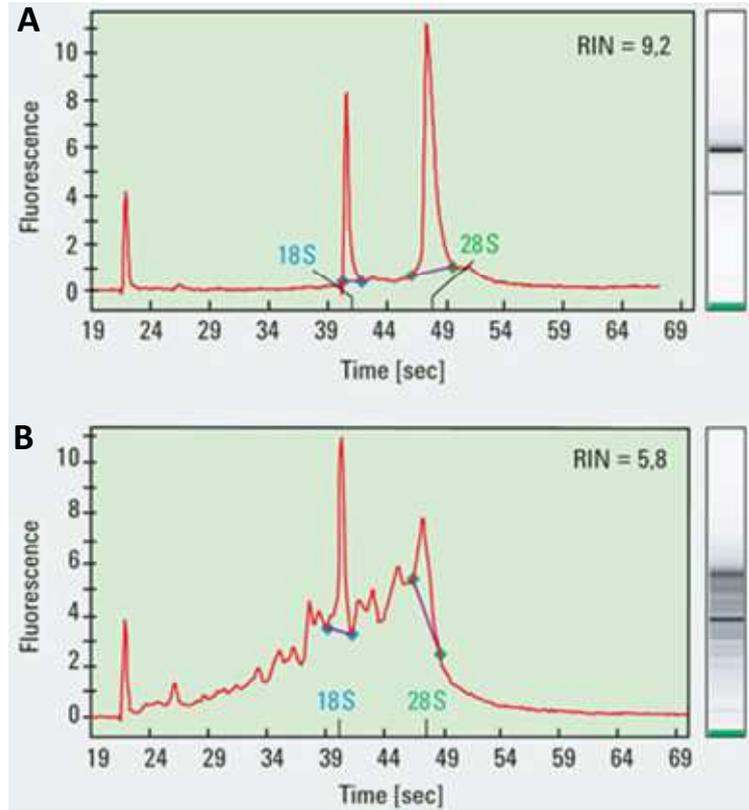


Figure 10. Electropherogram and gel-like image obtained with the Agilent 2100 Bioanalyser

(A) Analysis of a high quality RNA sample with a RIN of 9.2. (B) Analysis of a partially degraded RNA sample with a RIN of 5.8.

7. SYNTHESIS OF COMPLEMENTARY DNA

Complementary DNA (cDNA) was synthesized from 1 ug of RNA. RNA was diluted in H₂O DEPC in a final volume of 19 ul, and subsequently denaturalized at 65°C for 5 minutes. Meanwhile, 21 ul of premade cDNA mix (85.5 ul of 25 mM dNTPs (Roche Diagnostics GmbH, Mannheim, Germany), 21.5 ul of 0.1 mM DTT (Invitogen, Carlsbad, CA), 64 ul Hexanucleotide mix 10x (Roche Diagnostics GmbH, Mannheim, Germany), 428 ul 5x First strand buffer (Invitogen, Carlsbad, CA) and 401 ul H₂O DEPC) were supplemented with 1.4 ul of 200 U/ul Moloney-murine leukaemia virus reverse transcriptase (Invitogen, Carlsbad, CA) and 0.72 ul of 40 U/ul rRNAsin (Promega, Madison, WI). After the denaturalization of the 19 ul of RNA, 21 ul of the completed cDNA mix were added and incubated for 1 hour and 40 minutes at 37°C. The reaction was then stopped by increasing the temperature to 65°C for 10 minutes. The cDNA was immediately used, or stored at -20°C.

8. DETERMINATION OF THE MUTATIONAL STATUS OF THE IGHV GENE

The IGHV gene rearrangements were studied by polymerase chain reaction (PCR) in six independent reactions, one for each of the 6 IGHV subgroups, using sense primers complementary to the corresponding leader regions. The antisense primer used was complementary to the constant region, and was the same in the 6 reactions. Since CLL cells preferentially express IgM and IgD, the antisense primer used was complementary to IGHM. In the CLL cases that express IgG, the amplification of the IGHV rearrangement was achieved with the antisense primer against IGHG (BIOMED-2 protocol ²²²) (Figure 11).

SENSE PRIMERS:	
IGHV1 and 7:	5'- CTC ACC ATG GAC TGG ACC TGG AG -3'
IGHV2:	5'- ATG GAC ACA CTT TGC T(A/C)C AC(G/A) CTC -3'
IGHV3:	5'- CCA TGG AGT TTG GGC TGA GCT GG -3'
IGHV4:	5'- ACA TGA AAC A(C/T)C TGT GGT TCT TCC -3'
IGHV5:	5'- ATG GGG TCA ACC GCC ATC CT(C/T) G -3'
IGHV6:	5'- ATG TCT GTC TCC TTC CTC ATC TTC -3'
ANTISENSE PRIMERS:	
IGHM:	5'- GGA ATT CTC ACA GGA GAC GAG G -3'
IGHG:	5'- CTG AGT TCC ACG ACA CCG TCA -3'

Figure 11. Sense and antisense primers used in the amplification of the IGHV rearrangements.

The PCR mixes consisted in 2.5 ul of cDNA, 0.8 pmol/ul of sense and of antisense primer (Sigma-Aldrich Inc, St Louis, MO), 0.2 mM of dNTPs (Roche Diagnostics GmbH), 1.5 mM of MgCl₂ (Genecraft, Cologne, Germany), and 0.075 U/ul of Taq DNA Polymerase (SupraTherm™ Taq DNA polymerase, Genecraft, Cologne, Germany) in buffer solution (Reaction buffer solution, Genecraft), in a final volume of

25 ul. The PCRs were performed in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany); a denaturalization step of 1 minute at 95°C was made followed by 30 cycles of: 30 seconds at 94°C (denaturalization), 30 seconds at 55°C (annealing), and 30 seconds at 72°C (extension); a final step of extension of 7 minutes at 72°C was done. The PCR products were further analyzed in a gel of 2% in agarose in order to identify the IGHV subgroup in usage. The corresponding PCR product was subsequently purified with the PowerPrep™ Express PCR purification kit (Origene, Rockville, MD) according to manufacturer instructions.

Finally, the purified PCR product was sequenced based on the Sanger method using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction v3.1 (Applied Biosystems, Foster City, CA). The sequencing reaction mix consisted in 8 ul of the purified PCR product, 0.1875 uM of the corresponding IGHV subgroup leader primer, and 3 ul of the Big Dye premix (Applied Biosystems), in a final volume of 20 ul in BDT buffer solution (BDT buffer solution 5x = 400 mM Tris HCl + 10 mM MgCl₂, pH 9.0). The sequencing reaction was performed in an Eppendorf Mastercycler (Eppendorf); an initial denaturalization step of 3 minute at 94°C was made followed by 25 cycles of: 30 seconds at 96°C (denaturalization), 15 seconds at 50°C (annealing), and 4 minutes at 60°C (extension). The sequencing reaction product was purified. For that, 5 ul of 125 mM EDTA and 60 ul of 100% ethanol were mixed and incubated for 15 minutes at 4°C. The mix was further centrifuged for 15 minutes at 14,000 rpm at 4°C, and the supernatant was discharged. Afterwards, 60 ul of 70% ethanol were added, and the mix was centrifuged for 5 minutes at 14000 rpm at 4°C. The supernatant was discharged, and the sequencing reaction product was allowed to dry in the dark. Subsequently, it was stored at -20°C until being sequenced in an ABI Prism 3130XL Genetic Analyser (Applied Biosystems).

The nucleotide sequences were visualized with the Chromas Lite software (Technelysium Pty Ltd, Tewantin, Australia), and the analyses were performed with the

IMGT/V-QUEST software (Centre National de la Recherche Scientifique, Montpellier, France).²²³

The IGHV mutational load was calculated as the percentage of germline identity, which means the percentage of the nucleotides in the sequence identical to the germline sequence. The sequences were studied from the FR1 to the FR3. The IGHV genes were classified as mutated (<98% germline identity) and unmutated (100% germline identity) according to previous works.⁷

9. QUANTIFICATION OF BIM, GILZ, AND FKBP5 BY QRT-PCR

The levels of mRNA of the gene BIM, GILZ, and FKBP5 were determined by quantitative real time polymerase chain reaction (QRT-PCR). The TaqMan[®] Probe-base chemistry (Applied Biosystems) was used, and all probes were pre-developed TaqMan[®] assays. The assays for BIM, GILZ, and FKBP5 consisted in two unlabeled PCR primers and a FAM dye labeled TaqMan[®] MGB probe, and were respectively: Hs00197982_m1, Hs00608272_m1, and Hs01561001_m1. The Glucuronidase beta (GUS) gene was used as endogenous control, and its pre-developed assay consisted in two unlabeled PCR primers and a VIC dye labeled TaqMan[®] MGB probe. Of note, all the assays spanned an exon junction thus avoiding the detection of genomic DNA.

The QRT-PCR reactions were prepared in a final volume of 25 ul with 2 ul of cDNA, 12.5 ul of TaqMan[®] Universal MasterMix (Applied Biosystems, Branchburg, NJ), and 1.25 ul of the correspondent Custom TaqMan[®] assay. The QRT-PCR reactions were run in the ABI PRISM 7900HT sequence detection system (Applied Biosystems) and the following thermal cycling parameters were used: 2 minute at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

For each cDNA sample, they were performed three QRT-PCR reactions for the gene of interest and two reactions for the control gene. The respective averages of the Ct values of the gene of interest and of the control were considered. Only the determinations with standard deviation (SD) of the Ct values below 0.2 were validated.

The Ct value is the fractional cycle number at which the fluorescence passes the fixed threshold (Figure 12). The relative quantification of gene expression was made applying the comparative Ct method ($\Delta\Delta Ct$). Accordingly, the average Ct value of the gene of interest was normalized to the average Ct value of the endogenous control ($\Delta Ct = Ct(\text{gene of interest}) - Ct(\text{GUS})$). Then, the ΔCt value of the sample in study was normalized to the ΔCt value of a commercial sample (Human Reference

RNA, Applied Biosystems) used in all experiments ($\Delta\Delta Ct = \Delta Ct$ study sample - ΔCt commercial sample). The levels of mRNA expression were the result of the $2^{-\Delta\Delta Ct}$ and were given as arbitrary units (AU).

The induction of gene levels (fold change) was determined as the ratio between the levels of mRNA expression in the cells treated with dexamethasone and the levels of mRNA expression in the untreated cells.

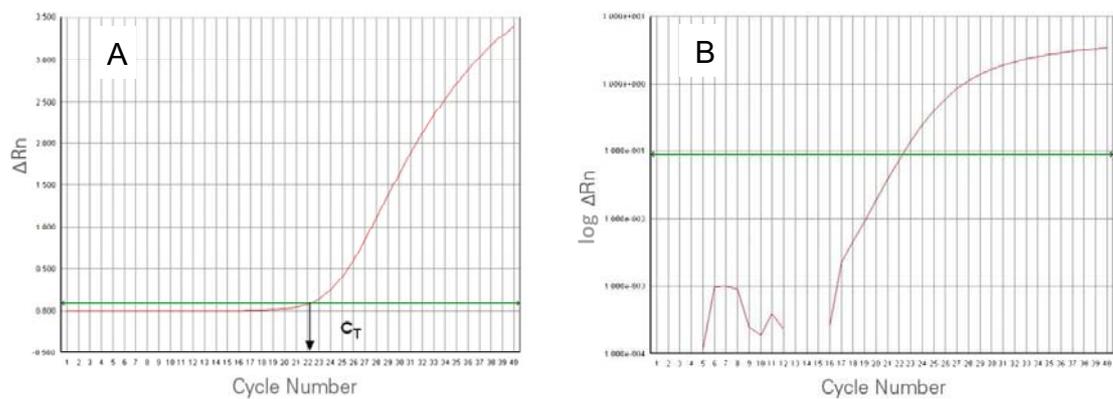


Figure 12. Analysis of the QRT-PCR data

ΔRn is the fluorescence of the reporter dye divided by the fluorescence of the passive reference dye ROX™ minus the baseline. Threshold is the average standard deviation of ΔRn for the early PCR cycles, multiplied by an adjustable factor. The threshold is represented by the green line. The fluorescence emitted by the gene of interest is represented by the red line. (A) ΔRn is plotted against PCR cycle number. The C_t value of the gene of interest is the cycle number at which the fluorescence emitted exceeds the threshold. (B) $\log (\Delta Rn)$ is plotted against the PCR cycle number. This representation shows the exponential growth of the PCR product and the threshold set in the exponential zone.

10. GENE EXPRESSION PROFILING ANALYSES

Gene expression profiling analyses were performed with the GeneChip® Human Genome U133 Plus 2.0 arrays (Affymetrix Inc, Santa Clara, CA). These arrays allow the analysis of transcription over the entire human genome with a single measurement. They consist in more than 54,000 probe sets that recognized over 47,000 transcripts and variants, including approximately 38,500 well characterized human genes. This array uses small oligos of 25 bp, and only a sample is studied per chip. There are 2 types of oligos, perfect match oligos, their nucleotide sequence is 100% homologue to the gene sequence, and mismatch oligos, which have a nucleotide change in the middle of the oligo sequence and are used to identify unspecific hybridizations. Each probe set of the array is formed by eleven pairs of perfect match oligos / mismatch oligos.

The first step in the microarray analysis is the preparation of the samples and of the poly-A RNA controls (Eukaryotic Poly-A RNA Control Kit, Affymetrix Inc) (Figure 13). For the sample, 2 ug of total RNA were reverse transcribed using a T7-Oligo(dT) Promoter Primer (One-Cycle cDNA Synthesis Kit, Affymetrix Inc), and the first strand cDNA was obtained. Then, it was synthesized the second cDNA strand (One-Cycle cDNA Synthesis Kit, Affymetrix Inc), and the double strand cDNA was cleanup (GeneChip Sample Cleanup Module, Affymetrix Inc). Next, cDNA was transcribed in biotin-labeled cRNA by means of an in vitro transcription reaction in the presence of T7 RNA Polymerase and of biotinylated nucleotide analog / ribonucleotide mix (GeneChip IVT labeling Kit, Affymetrix Inc). The biotin-labeled cRNA was cleaned-up and quantified, and subsequently fragmented by metal-induced hydrolysis (GeneChip Sample Cleanup Module, Affymetrix Inc).

The second step in microarray analysis is the hybridization of the biotin-labeled cRNA samples and controls (GeneChip® Hybridization Wash and Stain Kit, Affymetrix Inc).

Further steps consist in the setting up of the fluidics station (Fluidics Station 450/250, Affymetrix Inc), the washing and staining of the probe array, and the scanning (GeneChip®Scanner 3000 7G, Affymetrix Inc). All of the procedures were performed according to manufacturer recommendations. The GeneChip® Operating Software (GCOS) and the Affymetrix® Expression Console® software (Affymetrix Inc, Santa Clara, CA) were used.

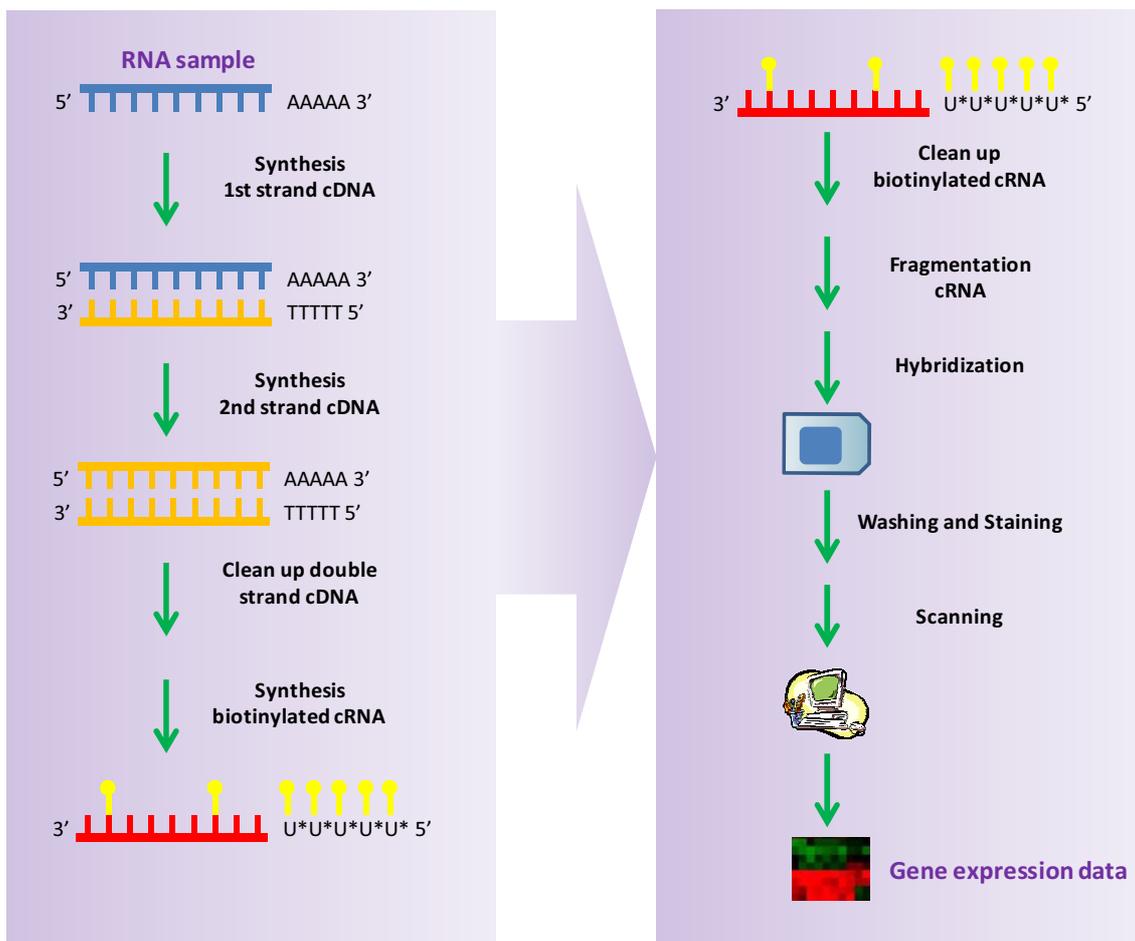


Figure 13. Schematic representation of the steps of the genome wide expression analysis.

The intensity data generated from scanning was subsequently processed. In this study, it was used the *RMA* (*fRMA*) methodology, a package running on R platform, which only takes in account the fluorescence emitted by the hybridization to

the perfect match oligos.²²⁴ Initially, the probe intensities were corrected against the background. Then, the fluorescent intensities were normalized in order to remove variations due to the preparation and hybridization of the samples. This approach allows further comparisons of data from different arrays. At last, the probe set intensities were summarized into probe set expressions.

The matrix with the probe set expressions data was filtered out for the probe sets with expression levels above 5, and was subsequently analyzed with the TM4 Software Suite.²²⁵ The unsupervised hierarchical clustering analysis of the data, in which the probe sets were grouped into clusters according to their pattern of expression, was performed.

Further, the statistical differential expression analysis was made. This is a supervised analysis, the samples are previously assigned to a group, and statistical tests are applied in order to retrieve the probe sets differently expressed between groups. It was used the *limma* package from the Bioconductor project, *limma* applies the empirical Bayes method to moderate the standard errors of the estimated log-fold changes.^{226;227} Only the changes in gene expression with a false discovery rate (FDR) value lower than 0.05 and a $\log\text{Ratio} > |0.75|$ were considered.

The online tool David was used for the functional annotation analysis based on Gene Ontology (GO).²²⁸ The lists of significant probe sets were also analyzed with IPA (Ingenuity® Systems, www.ingenuity.com).

11. STATISTICAL ANALYSES

For discrete parameters, in the descriptive statistics were included counts and frequency distributions. For quantitative variables, statistical measures included mean, medians, standard deviation, and range. The comparisons between groups were done with the Mann-Whitney test. Correlations between measures were performed using a parametric linear regression model, and Pearson correlation coefficients (R^2) were recorded. For all comparisons, P-values were two-sided, and the type I error was set at 5%. Statistical analyses were done with the use of SPSS v18.0 software (IBM, Somers, NY) and GraphPad Prism v5.0 software (La Jolla, CA).

RESULTS

Results

1. ANALYSIS OF THE RESPONSE OF CLL CELLS TO DEXAMETHASONE TREATMENT ACCORDING TO THE MUTATIONAL STATUS OF THE IGHV GENES AND THE EXPRESSION OF ZAP70

Glucocorticoids are able to induce the apoptosis of CLL cells. Recent reports have shown that CLL cells from patients with unmutated IGHV genes / high ZAP70 expression show better responses to the *in vitro* treatment with prednisolone or methylprednisolone than cells from patients with mutated IGHV genes / low ZAP70 expression.²⁰⁹⁻²¹² Although it was expected that CLL cells treated with other glucocorticoids would show different response according to the mutational status of the IGHV genes or the expression of ZAP70, a series of CLL cases was study for the response to the *in vitro* treatment with dexamethasone.

Samples from 50 patients diagnosed with CLL were selected. The percentage of CLL cells in the PBMC samples and the viability of the cells were determined after the recover from thawing in culture for 1 hour. The mutational status of the IGHV genes was determined, and the expression of ZAP70 was accessed by flow cytometry. The CLL cells were treated with dexamethasone for 24 hours, and the response was evaluated. The main characteristics of the series are summarized in Table 4.

Results

Table 4. Clinic-biological characteristics and response to the treatment with dexamethasone of the series of patients with CLL

Sample number	Gender	Age (years)	13q14 deletion	11q22-q23 deletion	17p13 deletion	trisomy 12	ZAP70 (%)	IGHV category	CLL cells (%)	Cell viability (%)	Live cells (%)	Binet stage	TPS
1	M	71	yes	no	no	no	6	MCLL	86	67	94	A	no
2	F	71	no	no	no	no	7	MCLL	80	85	96	A	no
3*	F	44	yes	no	no	no	6	MCLL	90	52	100	A	no
4	M	69	no	no	no	yes	2	MCLL	73	71	93	A	no
5	M	70	no	no	no	no	10	MCLL	53	60	81	A	no
6	M	60	yes	no	no	no	5	MCLL	85	78	78	A	no
7	M	64	yes	no	no	no	1	MCLL	84	86	94	A	yes
8	M	65	yes	no	no	no	4	MCLL	82	79	89	A	no
9*	M	71	no	no	no	no	2	MCLL	85	65	100	A	no
10	F	49	yes	no	no	no	11	MCLL	76	53	89	A	no
11	M	48	yes				0	MCLL	86	73	84	A	no
12	F	40	yes	no	no	no	6	MCLL	78	76	76	A	yes
13*	F	47	yes	no	no	no	2	MCLL	81	72	99	A	yes
14	F	50	no	no	no	no	6	MCLL	72	73	90	A	no
15	M	41	yes	no	no	no	15	MCLL	90	76	49	B	yes
16	M	56	no	no	no	no	3	MCLL		83	78	A	no
17	M	69	no	no	no	no	0	MCLL	73	77	75	A	no
18	M	62	yes	no	no	no	4	MCLL		75	74	A	no
19	M	45	yes	no	no	no	2	MCLL	93	90	86	C	yes
20*	M	63	no	no	no	no	3	MCLL		57	85	A	no
21*	M	68	yes	no	no	no	6	MCLL	78	78	91	A	no
22	M	56	no	no	no	no	1	MCLL	65	89	86	A	no
23	F	76	yes	no	no	no	12	MCLL	64	68	74	A	no
24	M	53	yes	no	no	no	13	MCLL	68	86	93	A	no
25	M	58	yes	yes	yes	no	77	UCLL	80	83	52	A	no
26*	M	30	no	no	no	no	73	UCLL	83	72	53	A	yes
27*	F	60	no	no	no	no	35	UCLL	89	61	66	A	no
28	M	46	no	no	no	no	60	UCLL	64	80	83	B	yes
29	M	57	no	yes	no	no	64	UCLL	92	72	70	A	no
30*	M	55	no	no	no	yes	60	UCLL	83	72	64	A	yes
31*	M	54	yes	no	no	no	36	UCLL	81	87	52	A	no
32	M	72	no	no	no	no	30	UCLL	91	84	61	A	yes
33	M	74	no	no	no	yes	90	UCLL	89	70	89	A	yes
34*	M	61	yes	no	yes	no	51	UCLL	88	83	42	A	yes
35*	M	61	yes	no	no	no	51	UCLL	95	84	56	A	yes
36	M	49	no	no	no	no	39	UCLL	87	80	79	A	no
37	F	70	no	no	no	yes	73	UCLL	84	65	86	A	no
38	F	48	no	yes	no	no	30	UCLL	82	53	50	B	no
39	M	48	yes	no	no	no	69	UCLL	84	80	64	A	yes
40	F	41	yes	no	no	no	70	UCLL	94	79	66	A	yes
41	M	46	no	no	no	yes	73	UCLL	83	92	68	A	yes
42	M	58	no	no	no	no	70	UCLL	94	89	97	A	no
43*	F	69	yes	no	no	no	75	UCLL	90	91	66	A	yes
44	M	45	yes	no	no	no	49	UCLL	95	83	75	B	yes
45	M	79					26	UCLL	90	90	67	A	no
46	M	82	yes	no	no	no	46	UCLL	80	88	83	B	yes
47	M	56	yes	no	no	no	5	UCLL	87	84	79		
48 [†]	F	63	yes	no	yes	no	1		94	89	80	A	yes
49	F	54	yes	no	yes	no	59		94	67	84	B	no
50	M	67	yes	no	yes	yes	30		64	87	69	A	no

M: male; F: female; MCLL: CLL case with mutated IGHV gene; UCLL: CLL case with unmutated IGHV gene; Live cells (%): percentage of live cells after treatment with dexamethasone relative to the percentage of live cells in the untreated cells; TPS: treatment prior to sampling; *CLL case selected for GEP analysis; [†] CLL case with 17p13 deletion, low ZAP70 expression, and poor response to dexamethasone.

The median age at diagnosis was 58 years (range, 30-82 years), and there was a male predominance (72%). The mean percentage of CLL cells found in the PBMCs was 83% \pm 10 (mean percentage of CLL cells \pm SD), and the mean percentage of cell viability after thawing in this series was 77% \pm 11 (mean percentage of cell viability \pm SD). Binet clinical stage at diagnosis was known for 49 out of the 50 patients: 85.7% of the patients were stage A, 12.2% stage B, and 2% stage C. For 30 patients, samples were obtained before the onset of treatment. The ZAP70 expression was considered high in 48% of the patients. The IGHV mutational status was assessed in 47 cases, and 23 of them (49%) have unmutated IGHV genes (UCLL). All the cases having mutated IGHV genes (MCLL) had low ZAP70 expression, whereas only one UCLL case showed a low expression of ZAP70. Thus, and as described before,⁵⁷ the ZAP70 expression and the mutational status of the IGHV genes were correlated in this CLL series, $R^2=0.918$. FISH analyses of the main CLL chromosomal abnormalities were performed in 48 out of 50 patients, at the time the samples were obtained. According to the hierarchical model,⁸ 45.8% of the patients showed isolated 13q14 deletion, 10.4% 17p13 deletion, 10.4% trisomy 12, 4.2% 11q22-q23 deletion, and 29.2% presented no abnormality.

After 24 hours of treatment with 13.25 μ M dexamethasone, the percentage of live cells relative to untreated cells ranged from 42% to 100%. Notably, UCLL cases (n=23) had a significantly better response to dexamethasone than MCLL cases (n=24) (mean percentage of live cells \pm SD: 68% \pm 14.0 vs 85% \pm 11.3; $P<0.001$; Figure 14A). In agreement, the response to dexamethasone was also better in cases with high ZAP70 expression (n=24) than in those with low ZAP70 (n=26) (mean percentage of live cells \pm SD: 68% \pm 13.9 vs 85% \pm 11.0; $P<0.001$; Figure 14B).

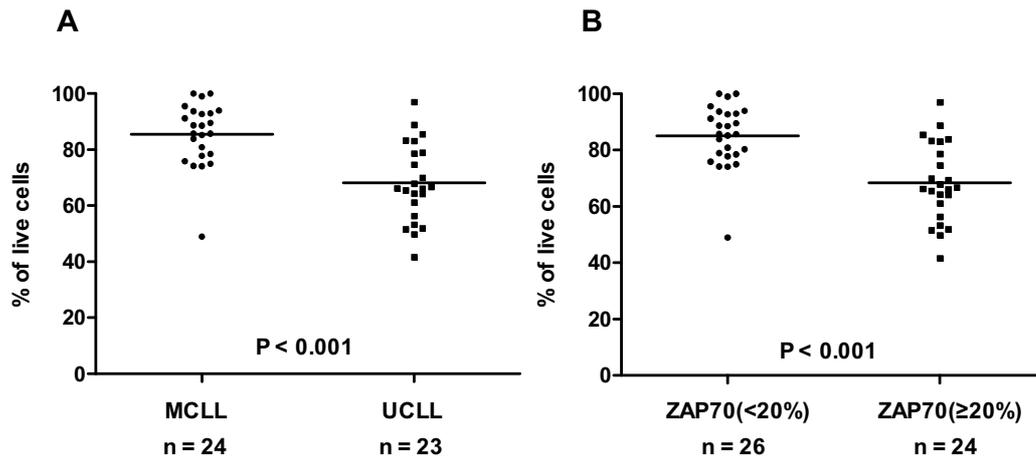


Figure 14. Response to dexamethasone according to the mutational status of the IGHV genes and to the expression of ZAP70

CLL cells were treated with 13.25 μ M dexamethasone for 24 hours, and the percentage of live cells was determined by annexinV / PI staining. (A) Response to dexamethasone in UCLL and in MCLL. UCLL cases show significantly higher response to dexamethasone in terms of percentage of live cells than MCLL cases. (B) Response to dexamethasone in high and low ZAP70 expression groups. CLL cases with high ZAP70 expression have better response to dexamethasone than cases with low ZAP70. Horizontal bars represent the mean values of live cells.

2. ANALYSIS OF THE RESPONSE OF CLL CELLS TO DEXAMETHASONE ACCORDING TO HIGH-RISK GENETIC ABNORMALITIES

Deletions or mutations in TP53 are related with resistance to many chemotherapy agents since those agents induce apoptosis through DNA damage.²²⁹ Likewise, alterations in other components of the DNA damage response pathway like ATM, have been associated with treatment resistance.²²⁹

It has been shown that glucocorticoids induced apoptosis independently of the DNA damage response pathway,²⁰² thus CLL cells with deletions in 17p13 (TP53) and 11q22-q23 (ATM) should present equivalent responses to dexamethasone as the cells without those abnormalities. Remarkably, the cases with 17p13 and 11q22-q23 deletions (n=7) had even better responses to dexamethasone than the cases without these high-risk genetic abnormalities (n=41) (mean percentage of live cells \pm SD: 64% \pm 16.2 vs 79% \pm 13.9; P=0.026) (Figure 15A).

Of note, the only case with 17p13 deletion and low ZAP70 expression disclosed a poor response to dexamethasone (sample number 48, Table 4). Moreover, after excluding the cases with high-risk genetic abnormalities (17p13 and 11q22-q23 deletions), ZAP70 expression retained its predictive value for the response to dexamethasone (mean percentage of live cells \pm SD: high ZAP70 (n=17) 71% \pm 13.1 vs low ZAP70 (n=24) 85% \pm 11.4; P=0.001) (Figure 15B).

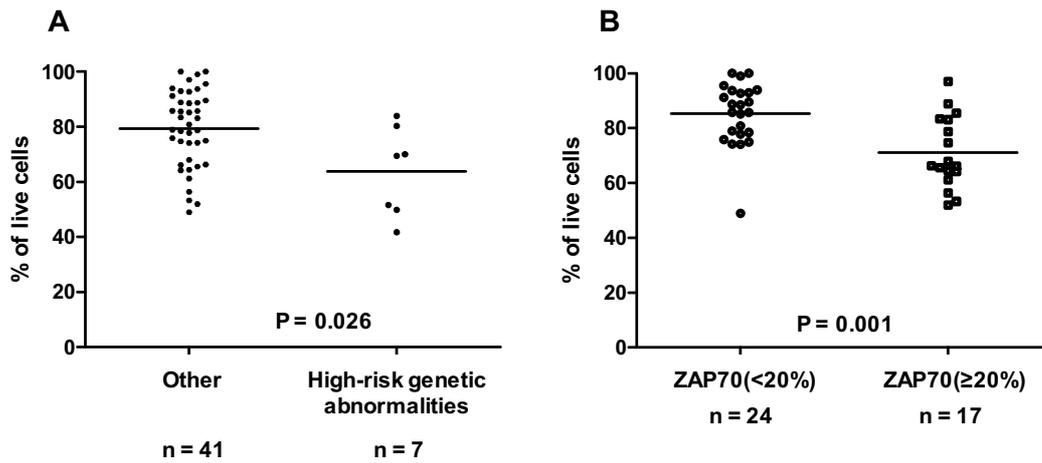


Figure 15. Response to dexamethasone according to the presence of genetic abnormalities

CLL cells were treated with 13.25 μ M dexamethasone for 24 hours, and the percentage of live cells was determined by annexinV / PI staining. (A) Response to dexamethasone in CLL cases with high-risk genetic abnormalities (17p13 and 11q22-q23 deletions) and in cases without high-risk genetic abnormalities (Other). CLL cases with high-risk genetic abnormalities show significantly higher response to dexamethasone, in terms of percentage of live cells, than the CLL cases without high-risk genetic abnormalities. (B) Response to dexamethasone in high and low ZAP70 expression groups of CLL cases without high-risk genetic abnormalities. CLL cases without high-risk genetic abnormalities and with high ZAP70 expression have better response to dexamethasone than cases without high-risk genetic abnormalities and with low ZAP70 expression. Horizontal bars represent the mean values of live cells.

3. INDUCTION OF BIM EXPRESSION BY DEXAMETHASONE

The expression of BIM has been reported to be induced by dexamethasone, at both mRNA and protein level, in different cellular models including in CLL cells.^{161;204;230-232} BIM is a BH3-only pro-apoptotic protein and a downstream mediator of dexamethasone induced cell death. Therefore, the magnitude of the response to dexamethasone and the degree of BIM induction should be related. To ascertain this reasoning, the degree of BIM induction was evaluated by QRT-PCR in 43 CLL samples after 24 hours of dexamethasone treatment. The response to dexamethasone was also determined after 24 hours by flow cytometry; results are shown in Table 5.

Table 5. Induction of BIM mRNA expression follow dexamethasone treatment

Sample number	ZAP70 category	IGHV category	Live cells (%)	BIM FC	Sample number	ZAP70 category	IGHV category	Live cells (%)	BIM FC
1	low	MCLL	94	1.77	24	low	MCLL	93	2.45
2	low	MCLL	96	2,55	25	high	UCLL	52	3.32
3	low	MCLL	100	2.06	26	high	UCLL	53	5.86
4	low	MCLL	93	1.88	27	high	UCLL	66	3.36
5	low	MCLL	81	1.89	28	high	UCLL	83	2.51
6	low	MCLL	78	3.53	29	high	UCLL	70	4.08
7	low	MCLL	94	3.39	30	high	UCLL	64	4.38
8	low	MCLL	89	3.1	31	high	UCLL	52	5.66
9	low	MCLL	100	1.72	32	high	UCLL	61	2.08
10	low	MCLL	89	2.5	33	high	UCLL	89	1.24
11	low	MCLL	84	2.2	34	high	UCLL	42	3.07
12	low	MCLL	76	2.33	35	high	UCLL	56	5.35
13	low	MCLL	99	1.37	36	high	UCLL	79	4.2
14	low	MCLL	90	2.53	37	high	UCLL	86	1.7
15	low	MCLL	49	3.92	38	high	UCLL	50	2.33
16	low	MCLL	78	1.78	39	high	UCLL	64	6.28
17	low	MCLL	75	2.99	40	high	UCLL	66	2.71
18	low	MCLL	74	3.46	42	high	UCLL	97	1.92
19	low	MCLL	86	3.68	43	high	UCLL	66	2.68
20	low	MCLL	85	2.14	44	high	UCLL	75	8.57
21	low	MCLL	91	2.22	47	low	UCLL	79	4.14
23	low	MCLL	74	3.01					

MCLL: CLL case with mutated IGHV gene; UCLL: CLL case with unmutated IGHV gene; Live cells (%): percentage of live cells after treatment with dexamethasone relative to the percentage of live cells in the untreated cells; BIM FC: BIM fold change. Sample number according to Table 4.

The levels of BIM mRNA increased after the treatment of CLL cells with dexamethasone (range from 1.24 to 8.57) and the degree of BIM induction was higher in those cases with higher levels of cell apoptosis. An inverse correlation between BIM induction and the percentage of live cells was observed ($P=0.001$; Figure 16). It is important to note that in all cases the treatment with dexamethasone induced the expression of BIM, even in those with poor or null response to dexamethasone, evaluated at 24 hours of treatment.

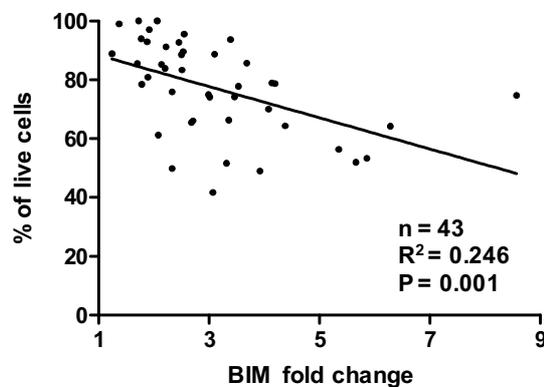


Figure 16. Correlation between the induction of BIM and the response to dexamethasone

CLL cells were treated with 13.25 μM of dexamethasone for 24 hours and then both BIM fold change and response to treatment with dexamethasone were determined. The scatter-plot shows a linear correlation between induction of BIM and response to dexamethasone.

Moreover, the CLL cases with unmutated IGHV genes ($n=20$) showed higher levels of BIM induction than the cases with mutated IGHV genes ($n=23$) (mean BIM fold change $\pm\text{SD}$: 3.77 ± 1.84 vs 2.54 ± 0.71 ; $P=0.018$; Figure 17A). As well, the levels of BIM induction were higher in the group of CLL cases with high ZAP70 expression ($n=19$) than in the group with low ZAP70 ($n=24$) (mean BIM fold change $\pm\text{SD}$: 3.75 ± 1.89 vs 2.61 ± 0.78 ; $P=0.042$; Figure 17B). These findings are in agreement with the better response to dexamethasone observed in the CLL cases with unmutated IGHV genes and high ZAP70 expression.

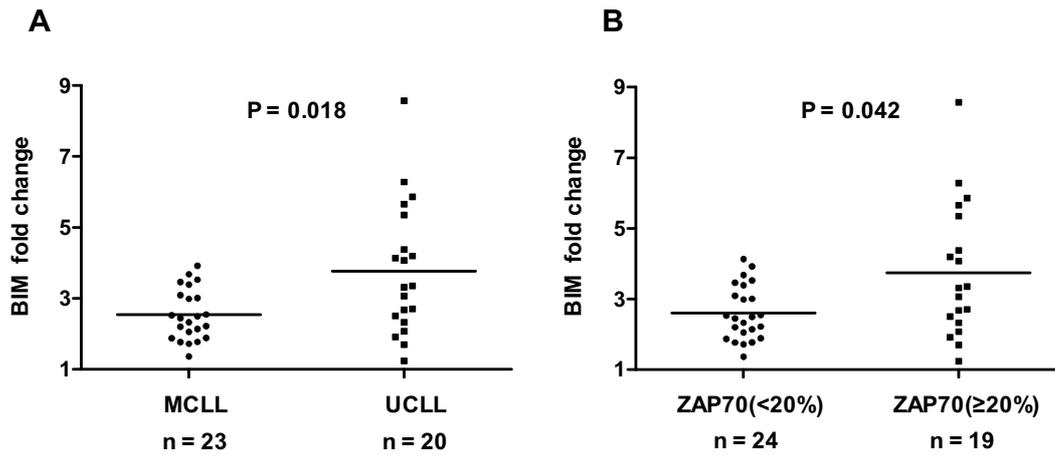
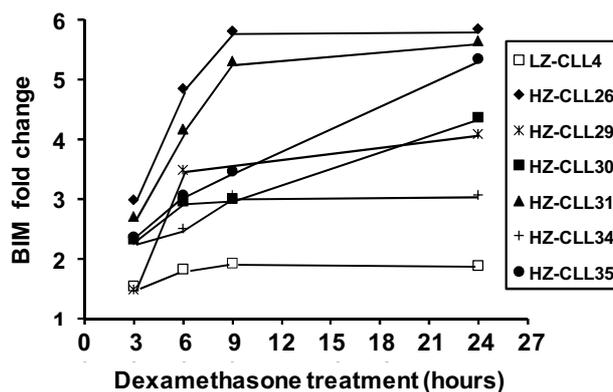


Figure 17. Induction of BIM in CLL cells according to the mutational status of the IGHV genes and the expression of ZAP70

CLL cells from 43 cases were treated with 13.25 μM of dexamethasone for 24 hours, and afterwards both BIM fold change and response to dexamethasone treatment were determined. (A) CLL cases with unmutated IGHV (UCLL) genes have a significantly higher induction of BIM than the cases with mutated IGHV genes (MCLL). (B) CLL cases with high ZAP70 expression have a significantly higher induction of BIM than the cases with low ZAP70. Horizontal bars represent the mean values of BIM induction.

The presented results were indicative that the different responses to dexamethasone observed among CLL cases are due to events occurring before BIM. In order to determine the best time point to identify the genes regulated by dexamethasone that acted upstream BIM, the kinetics of BIM induction was studied. For that, the levels of BIM mRNA were analyzed by QRT-PCR at different time points, in primary cells from 7 patients with CLL (Figure 18).



Time (h)	BIM fold change						
	LZ-CLL4	HZ-CLL26	HZ-CLL29	HZ-CLL30	HZ-CLL31	HZ-CLL34	HZ-CLL 35
3	1.55	2.99	1.49	2.33	2.71	2.31	2.36
6	1.82	4.86	3.48	2.97	4.17	2.5	3.07
9	1.92	5.82	4.08	3.01	5.31	3.07	3.46
24	1.88	5.86	4.08	4.38	5.66	3.07	5.35

Figure 18. Time-course of the induction of BIM after treatment with dexamethasone

CLL cells from 7 cases were treated with 13.25 μ M dexamethasone and BIM levels were evaluated at 3, 6, 9, and 24 hours by QRT-PCR. Results are expressed as the BIM fold change. HZ-CLL stands for high ZAP70 expression and LZ-CLL for low ZAP70 expression; the number after CLL is the sample number according to table 4 and 5. The induction of BIM is high in the initial hours of treatment with dexamethasone and stabilizes after 9 hours.

As early as after 3 hours of treatment, an increase in BIM mRNA was already detected. In five of the cases, levels kept increasing up to 9 hours, and then remained stable, whereas in the other two cases, an additional increase in BIM levels was observed from 9 to 24 hours. The time point 6 hours was selected for further studies since it preceded the highest levels of BIM induction observed after dexamethasone treatment.

4. GENE EXPRESSION PROFILING ANALYSES OF CLL SAMPLES TREATED WITH DEXAMETHASONE

GEP analyses were performed in a series of CLL samples to identify genes potentially implicated in the differential response to dexamethasone. For this, 7 CLL samples with high ZAP70 expression and 5 with low ZAP70 expression were selected (Table 4). Tumor cells were treated with dexamethasone or left with standard medium for 6 hours, and total RNA was extracted and further processed.

The unsupervised analysis of the expression data was performed using the 1,000 probe sets showing the highest variability. The sample pairs, treated and untreated cells from the same patient, clustered together. Moreover, two main branches were defined, one included the cases with high ZAP70 expression, and the other the cases with low ZAP70 expression (Figure 19). This indicated that the different responses to dexamethasone observed between the CLL cases with high ZAP70 expression and with low ZAP70 expression were reflected in GEP.

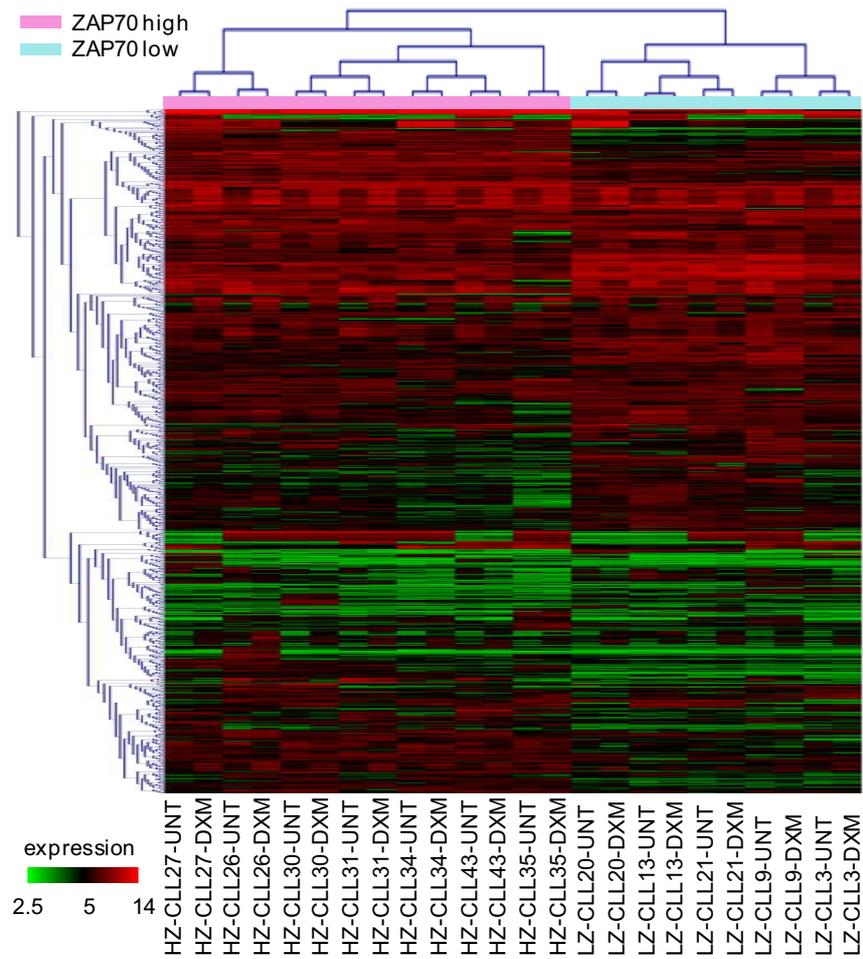


Figure 19. Unsupervised analysis of the 1,000 probe sets with the most variable expression

Dendrogram representing the unsupervised analysis of the 1,000 probe sets with the most variable expression applying the hierarchical clustering algorithm. DXM stands for dexamethasone treated cells, and UNT stands for untreated cells. HZ stands for high ZAP70 expression, and LZ stands for low ZAP70 expression; the number after CLL is the sample number according to Table 4.

4.1. Independent analyses of gene expression profiling of the CLL groups defined by ZAP70 expression

The effect of dexamethasone treatment was independently analyzed in the high and low ZAP70 groups by means of supervised analysis. It has been found that dexamethasone treatment up-regulated the expression of 314 probe sets (153 genes) in the group with high ZAP70 expression, whereas in the low ZAP70 group a total of 226 probe sets (118 genes) resulted up-regulated (Appendix 1). Moreover, dexamethasone treatment induced the down-regulation of 219 probe sets (153 genes) in CLL cases with high ZAP70 expression, and of 222 probe sets (155 genes) in cases with low ZAP70 expression (Appendix 2).

The list of the probe sets up-regulated in the cases with high ZAP70 expression was compared with the list of probe sets up-regulated in the cases with low ZAP70 expression, and additional lists with the common and uncommon up-regulated probe sets were retrieved. The same approach was done with the lists of down-regulated probe sets. Next, these lists of probe sets were separately analyzed for functional annotation using gene ontology (GO) categories for “biological processes” allowing for the discovery of overrepresented categories of genes.

Functional annotation analysis of the up-regulated genes revealed that the most significant GO categories in the high and low ZAP70 groups were related to apoptosis, although the high ZAP70 group presented more probe sets (18 genes) in the terms related to apoptosis than the low ZAP70 group (15 genes) (Figure 20).

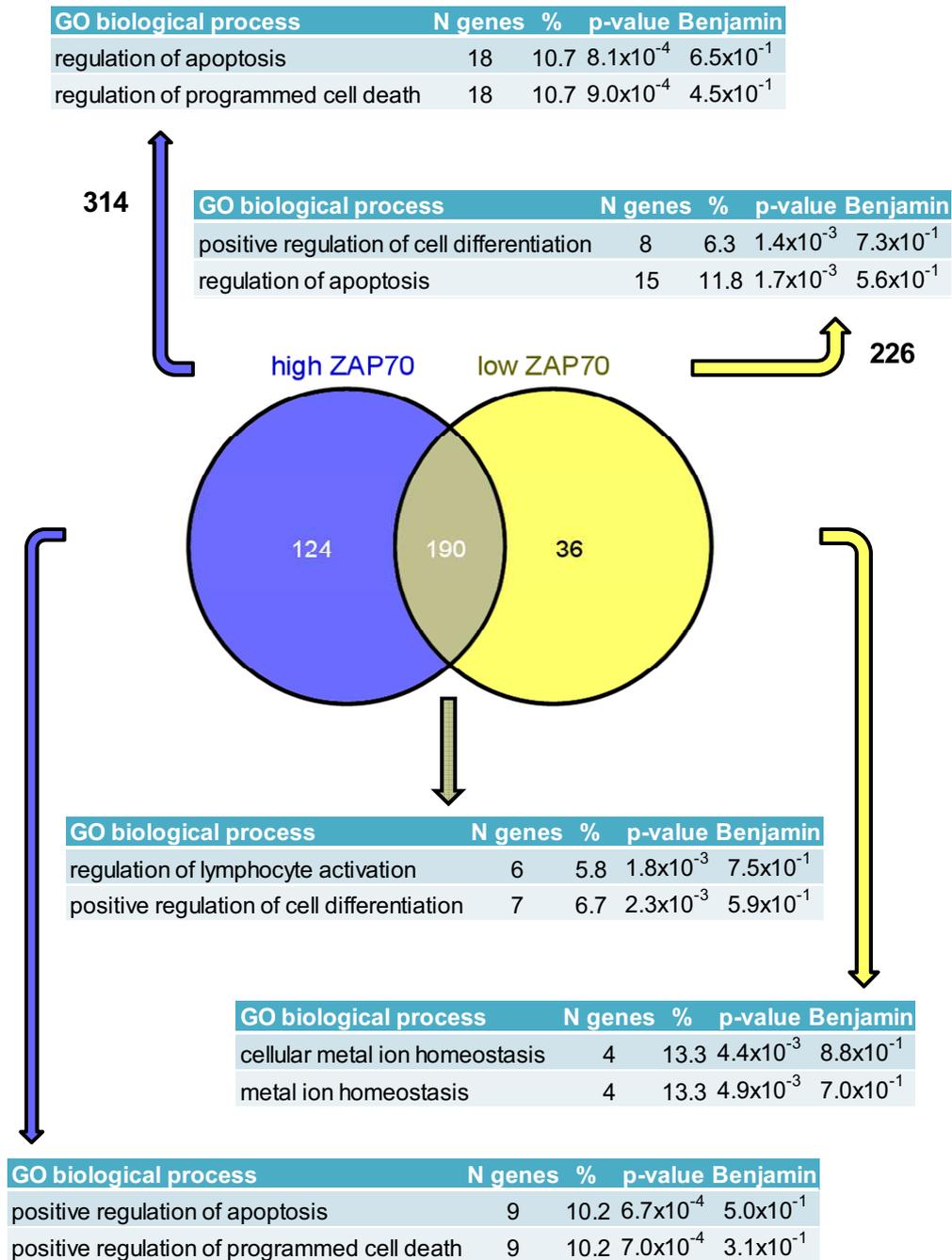


Figure 20. Most significant biological processes targeted by the genes up-regulated by dexamethasone according to ZAP70 expression groups

Among the upregulated genes, 190 probe sets were shared by both ZAP70 expression groups, whereas 124 probe sets were only up-regulated in samples with high ZAP70 expression, and 36 probe sets were only up-regulated in samples with low ZAP70 expression. Interestingly, the analysis of the common 190 probe sets showed

that the most enriched category was *regulation of lymphoid activation*, which included genes such as IL7R and CTLA4. Of note, analysis of the 124 probe sets solely up-regulated in samples with high ZAP70 expression showed a significant enrichment in genes involved in *positive regulation of apoptosis*, whereas analysis of the 36 probe sets only up-regulated in cases with low ZAP70 disclosed that the most enriched GO category was related to *ion homeostasis*. This latter term includes genes that participate in any process involved in the maintenance of an internal steady state of metal ions at the level of a cell, thus the relevance of apoptosis in this subgroup was less notorious. Finally, enrichment in the GO category *apoptosis* was observed in the analysis of both high and low ZAP70 groups.

In summary, the treatment with dexamethasone induces the expression of more genes related to apoptosis in the cases with high ZAP70 expression, the ones with better response to dexamethasone. Moreover, the conjunctive analysis of the ZAP70 groups show that the genes up-regulated only in cases with high ZAP70 expression were related to apoptosis unlike the genes solely up-regulated in the cases with low ZAP70 expression.

GO analysis of down-regulated probe sets showed that in both high and low ZAP70 groups the most significant term was *immune response* (Figure 21).

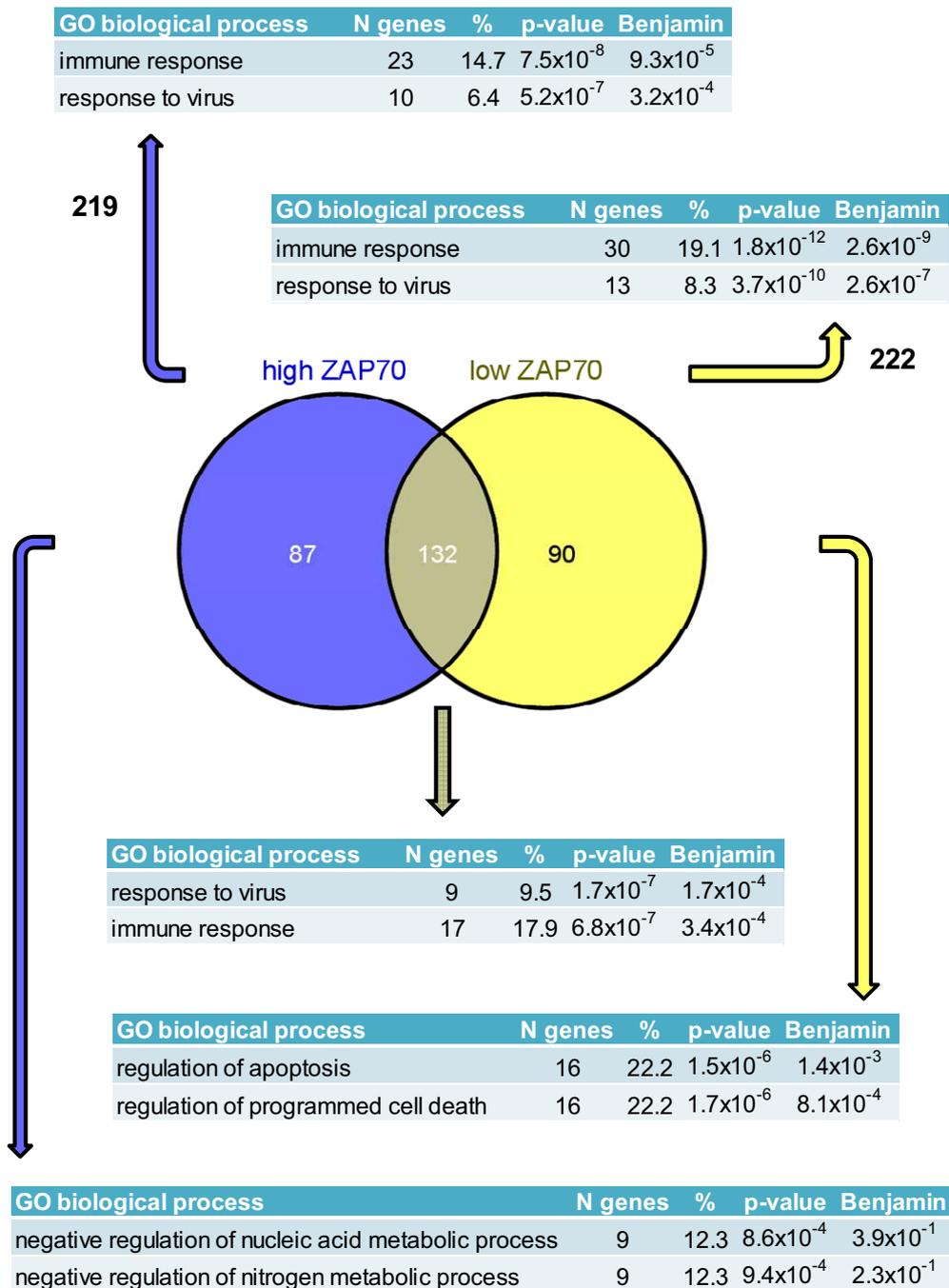


Figure 21. Most significant biological processes targeted by the genes down-regulated by dexamethasone according to ZAP70 expression groups

Among all the down-regulated genes, again the majority of the probe sets were common in both groups. A total of 132 probe sets were shared by both ZAP70 groups, and a significantly enrichment in genes belonging to the terms *response to virus* and *immune response* was observed. 80 probe sets were exclusively down-regulated in the

high ZAP70 group, and the most significant term was *negative regulation of nucleic acid metabolic process*. The probe sets that were exclusively down-regulated in CLL cases with low ZAP70 expression (n=90) were significantly enriched in genes related to *regulation of apoptosis*. Of note, the majority of them were involved in the positive regulation of apoptosis (as for example BID and TNF).

In summary, the genes down-regulated by dexamethasone appear to play a minor role in the induction of apoptosis. Interestingly, the genes solely down-regulated in the low ZAP70 group were inducers of apoptosis, which is in line with the lower responses to dexamethasone observed in these cases.

The top 10 probe sets with the highest variation caused by the treatment with dexamethasone were selected for each ZAP70 group (Table 6). The comparison of these probe sets lists showed that 3 genes were commonly up-regulated in high and low ZAP70 groups, namely FKBP5, DDIT4, and TMEM2. In addition, 4 genes were commonly down-regulated by dexamethasone in both ZAP70 expression groups: KMO, PALM2-AKAP2, IFIT2, and SAMD9L. Of note, FKBP5 was the most up-regulated gene in both ZAP70 groups, and was represented by three different probe sets.

Table 6. The top 10 most up-regulated and down-regulated probe sets in CLL groups with high and low ZAP70 expression caused by the treatment with dexamethasone

high ZAP70 expression group			
up-regulated			
Gene Symbol	Probe set	logRatio	FDR
FKBP5*	224856_at	3.247	3.49E-07
DDIT4*	202887_s_at	3.136	1.15E-07
FKBP5*	204560_at	3.073	7.45E-06
TMEM2*	218113_at	2.942	3.22E-08
TSC22D3	207001_x_at	2.790	9.49E-08
FKBP5*	224840_at	2.693	1.04E-08
TGFBR3	226625_at	2.525	9.77E-06
TGFBR3	204731_at	2.496	1.24E-04
C18orf1	242551_at	2.384	7.73E-09
---	242406_at	2.314	1.74E-08
down-regulated			
Gene Symbol	Probe set	logRatio	FDR
FCRL3	231093_at	-1.682	8.94E-06
KMO*	211138_s_at	-1.675	2.58E-07
AKAP2 /// PALM2-AKAP2*	226694_at	-1.639	4.50E-06
IFIT2*	226757_at	-1.628	3.15E-05
SETBP1	227478_at	-1.558	1.24E-05
AMIGO2	222108_at	-1.543	5.99E-06
BCL2A1	205681_at	-1.539	8.90E-06
KMO	205306_x_at	-1.522	1.97E-07
SAMD9L*	226603_at	-1.493	1.03E-05
AKAP2 /// PALM2-AKAP2*	202759_s_at	-1.480	2.27E-05

low ZAP70 expression group			
up-regulated			
Gene Symbol	Probe set	logRatio	FDR
FKBP5*	224840_at	2.917	9.51E-08
CD72	215925_s_at	2.671	7.55E-08
FKBP5*	224856_at	2.664	3.77E-05
TMEM2*	218113_at	2.490	3.20E-06
FKBP5*	204560_at	2.325	9.84E-04
DNMBP	212838_at	2.229	9.13E-08
HIPK2	225116_at	2.096	3.10E-05
---	215528_at	2.076	3.31E-07
DDIT4*	202887_s_at	2.005	1.30E-04
---	241893_at	1.964	1.37E-04
down-regulated			
Gene Symbol	Probe set	logRatio	FDR
CCL4	204103_at	-2.195	2.64E-06
AKAP2 /// PALM2-AKAP2*	202759_s_at	-1.726	6.46E-05
AKAP2 /// PALM2-AKAP2*	226694_at	-1.699	4.13E-05
ISG15	205483_s_at	-1.587	1.22E-03
SAMD9L*	226603_at	-1.516	1.01E-04
AKAP2 /// PALM2-AKAP2	202760_s_at	-1.494	7.21E-04
STAT1	AFFX-HUMISGF3A/M97935_MB_at	-1.406	1.81E-04
KMO*	211138_s_at	-1.395	2.70E-05
MIR21	224917_at	-1.392	1.90E-04
IFIT2*	226757_at	-1.379	1.27E-03

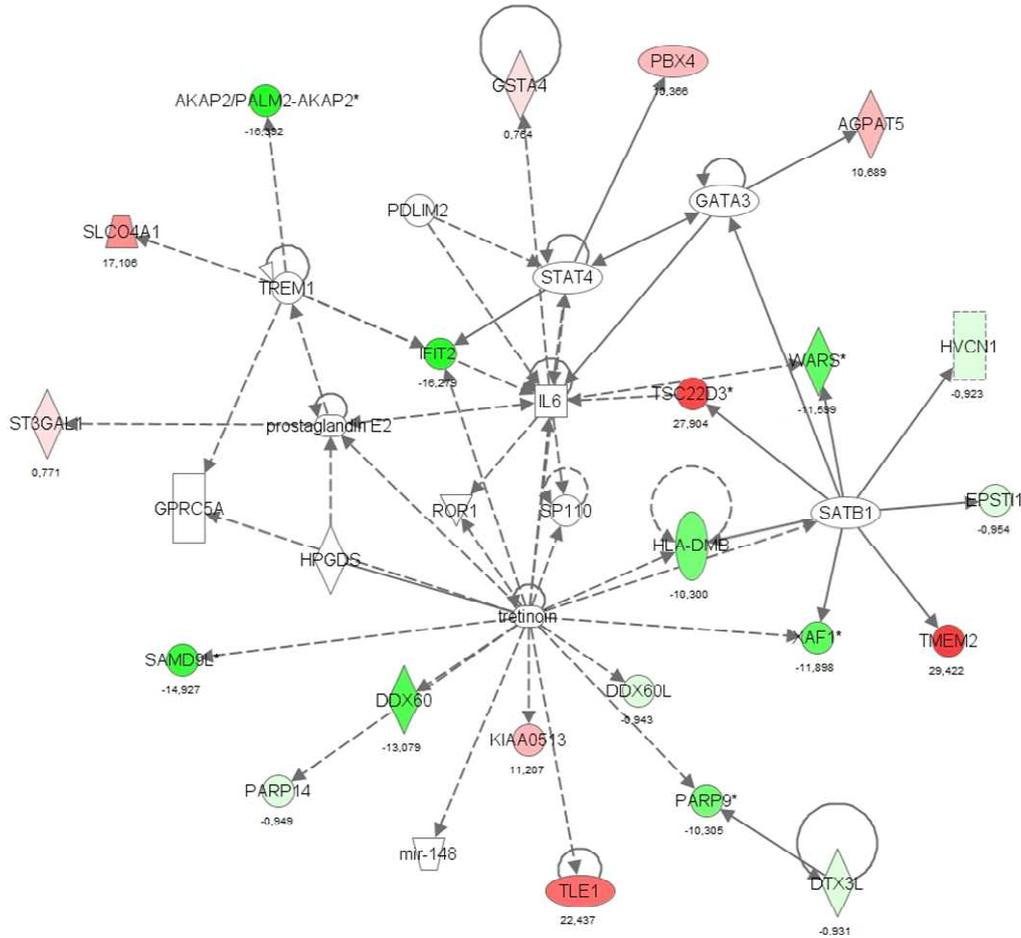
Genes are ranked according to their logRatio values calculated as the difference in log expression value using the untreated cells group as baseline. *common probe sets in high and low ZAP70 expression groups.

The lists of the significant probe sets were also analyzed with IPA. Two datasets were analyzed, the high ZAP70 expression group dataset and the low ZAP70 expression group dataset. Since IPA software is able to discriminate between increased and decreased expressions, the datasets included both up and down-regulated probe sets, and their respective logRatios (high ZAP70 group = 533 probe sets; low ZAP70 group = 448 probe sets).

The IPA software built networks that relate the genes present in the dataset with other genes based on extensive records maintained in the Ingenuity Pathways Knowledge Base (IPKB). The top IPA network obtained in the analysis of high ZAP70 group dataset had associated the functions of *cellular growth and proliferation*, *hematological system development and function*, and *tissue development* (Figure 22). This network included several of the top 10 most up-regulated and down-regulated probe sets, corresponding to the following genes: GILZ (alias TSC22D3), TMEM2, PALM2-AKAP2, IFIT2, and SAMD9L. Of note, some of these probe sets were also found in the top 10 most up-regulated and down-regulated genes of the low ZAP70 group (see Table 6).

Results

Network 1 : up and down ZAPhigh - 2012-05-09 07:29 PM : up and down ZAPhigh : up and down ZAPhigh - 2012-05-09 07:29 PM



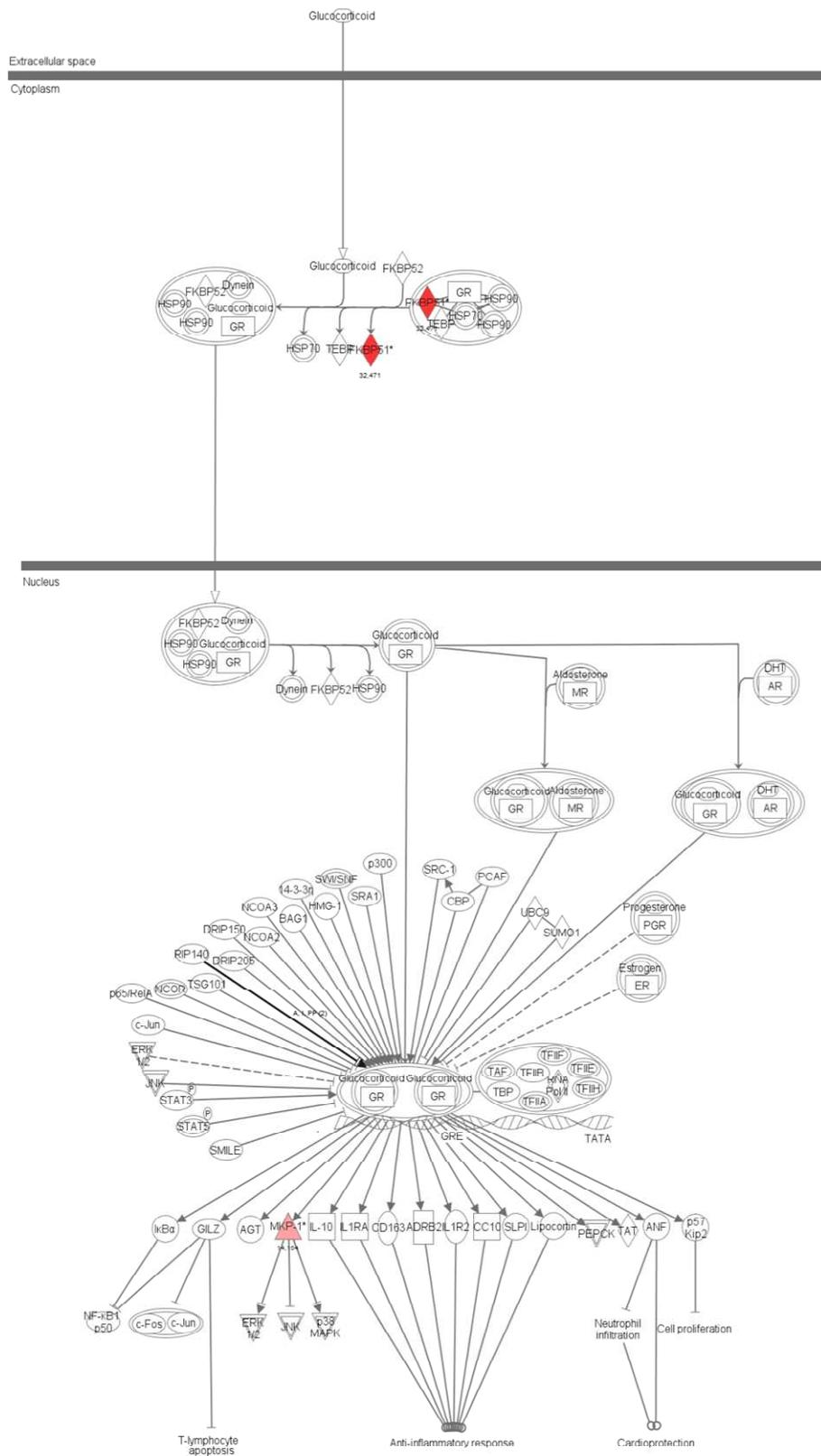
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Figure 22. Top IPA network obtained in the analysis of high ZAP70 group dataset

IPA tool was used to connect the dataset of the 533 probe sets from the high ZAP70 group based upon a database of published observations. The probe sets were mapped to the corresponding gene within IPKB. The represented pathway is the IPA network that includes the highest number of queried genes. Query genes are represented as color nodes, and the genes added by the program are represented as empty nodes. Color gradations are based upon gene regulation at the logRatio level. Red color: up-regulated gene; green color: down-regulated gene.

It were analyzed the genes regulated by dexamethasone in the high ZAP70 group that belonged to the IPA *canonical pathway of the glucocorticoid receptor signaling* (Figure 23).

Glucocorticoid Receptor Signaling



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Figure 23. Genes of the high ZAP70 group dataset belonging to the IPA *canonical pathway of the glucocorticoid receptor signaling*

IPA tool was used to retrieve the genes of the dataset of the 533 probe sets from the high ZAP70 group that belonged to the IPA *canonical pathway of the glucocorticoid receptor signaling*. The probe sets were mapped to the corresponding gene within IPKB. The represented pathway is a part of the IPA *canonical pathway of the glucocorticoid receptor signaling*. Color gradations are based upon gene regulation at the logRatio level. Red color: up-regulated gene.

The FKBP5 (alias FKBP51) gene was highlighted and it figured in the initial steps of the glucocorticoid pathway. FKBP5 gene codifies for a co-chaperone of the GR complex that maintains the receptor complex in the cytoplasm. After glucocorticoid binding, FKBP5 is replaced by FKBP4 which allows for the nuclear translocation of the GR complex.²³³

The previous analyses pointed that FKBP5 may play an important role in the response to dexamethasone. It was thought of interest to analyze the levels of FKBP5 in the untreated and in the treated cells of ZAP70 groups. For this, GEP of the untreated cells from the high ZAP70 group was compared with the GEP of the untreated cells from the low ZAP70 group by means of supervised analysis (Appendix 3). The GEPs of the treated cells from the high and low ZAP70 groups were also compared (Appendix 4).

The FKBP5 expression levels were higher in the untreated cells from the high ZAP70 group than in the untreated cells from the low ZAP70 group (Figure 24).

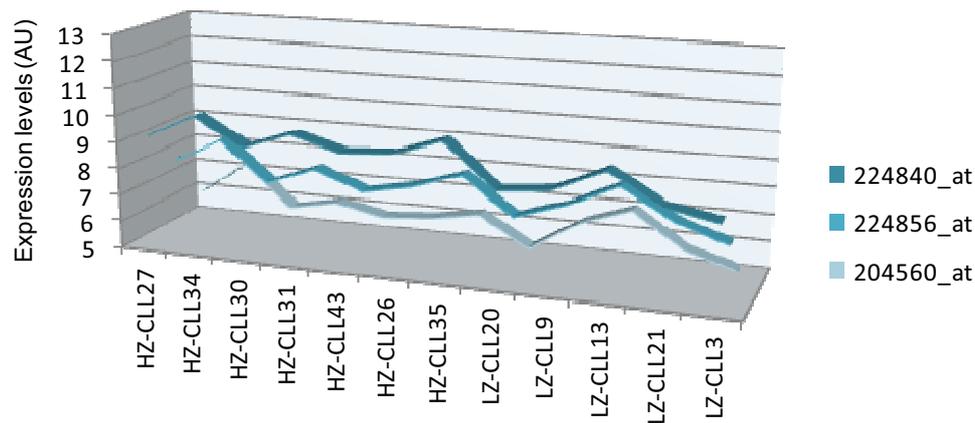


Figure 24. Expression levels of FKBP5 in the untreated cells

Graphic representation of the expression values of FKBP5 in the untreated cells of each CLL case studied. They are represented the FKBP5 expression values of the 3 probe sets of this gene previously found in the list of the top 10 most up-regulated probe sets in the CLL groups with high and low ZAP70 expression. FKBP5 expression values are given as arbitrary units (AU). HZ stands for high ZAP70 expression, and LZ stands for low ZAP70 expression; the number after CLL is the sample number according to Table 4.

Significant differences in the FKBP5 expression between the ZAP70 groups were observed for the probe set 224840_at (logRatio=0.958, FDR=0.0129). Moreover, the expression values of the probe sets 24856_at and 20560_at, tend to have highest expressions in the high ZAP70 group.

The comparison between the expression values of FKBP5 in the treated cells from the high ZAP70 expression group, and the expression values in the treated cells from the low ZAP70 group, revealed that the cells from the high ZAP70 group had higher levels of FKBP5 than the cells from the low ZAP70 group (Figure 25).

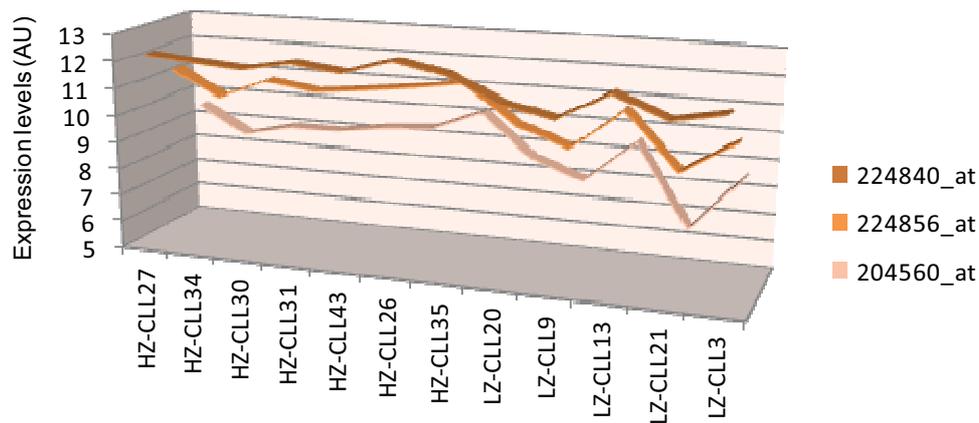


Figure 25. Expression levels of FKBP5 in the treated cells

Graphic representation of the expression values of FKBP5 in the treated cells of each CLL case studied. They are represented the FKBP5 expression values of the 3 probe sets of this gene previously found in the top 10 most up-regulated probe sets in CLL groups with high and low ZAP70 expression. FKBP5 expression values are given as arbitrary units (AU). HZ stands for high ZAP70 expression, and LZ stands for low ZAP70 expression; the number after CLL is the sample number according to Table 4.

The levels of expression of the probe set 224856_at were significantly higher in the high ZAP70 CLL cases than in the low ZAP70 cases ($\log\text{Ratio}=1.068$, $\text{FDR}=0.0416$). The probe sets 224840_at and 204560_at also showed higher expressions in the high ZAP70 group, although the differences between ZAP70 groups did not reach $\log\text{Ratio}>|0.75|$.

In summary, these results led us to hypothesize that the levels of FKBP5 could be involved in the different responses to dexamethasone observed in CLL cases. Consequently, FKBP5 was selected for further studies in a large CLL series.

4.2. Analysis of the genes with a significant differential regulation by dexamethasone

A supervised analysis was conducted in order to retrieve the genes that had a significant differential regulation by the treatment with dexamethasone in the two ZAP70 expression groups. For this, the interaction term was calculated by assessing the difference between the genes induced/repressed by dexamethasone in the low ZAP70 expression group, and the genes induced/repressed by dexamethasone in the high ZAP70 group:

$$\left[\left[\text{expression in treated cells of low ZAP70} \right] - \left[\text{expression in untreated cells of low ZAP70} \right] \right] - \left[\left[\text{expression in treated cells of high ZAP70} \right] - \left[\text{expression in untreated cells of high ZAP70} \right] \right]$$

Considering P-values lower than 0.001, 45 probe sets (38 genes) were identified as differently regulated in the two ZAP70 expression groups (Figure 26).

Results

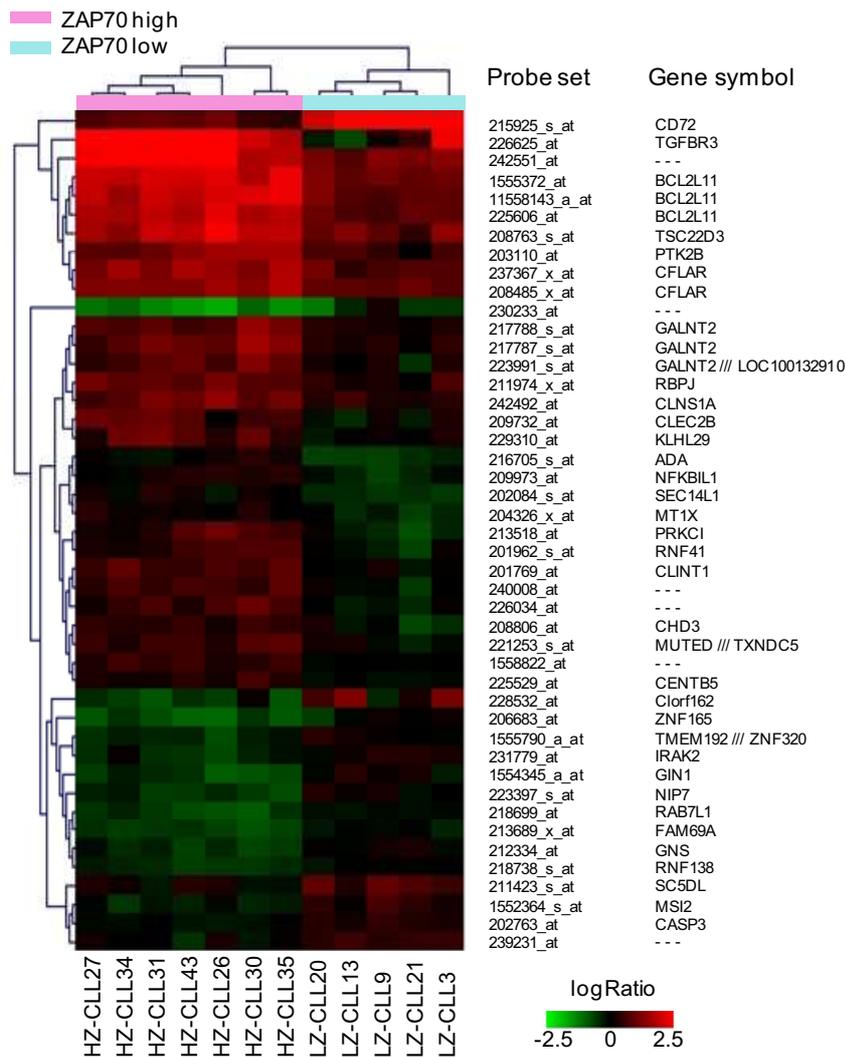


Figure 26. Unsupervised cluster analysis of the 45 probe sets retrieved in the analysis of the interaction term.

For each probe set, changes in expression due to dexamethasone treatment are displayed as LogRatios. HZ stands for high ZAP70 expression, and LZ stands for low ZAP70 expression; the number after CLL is the sample number according to Table 4.

The unsupervised cluster analysis of the 45 probe sets differently regulated in the ZAP70 groups highlighted that the pro-apoptotic gene BIM (alias BCL2L11) clustered with GILZ (alias TSC22D3). This was indicative that the two genes were altered in a similar way by dexamethasone.

It was performed the functional annotation analysis of the 45 probe set list. The GO analysis revealed a significant enrichment in genes related to *regulation of apoptosis*, which means that the two ZAP70 groups differently regulated genes involved in the apoptosis, in the consequence of the treatment with dexamethasone. This was in line with the results of the GO analyses of the individual probe set lists of the CLL groups defined by ZAP70 expression where it was observed that the high ZAP70 group presented genes related to apoptosis not present in the low ZAP70 group.

Moreover, the results of the GO analyses of the interaction term probe set list strengthened the observations made at the time of the comparison of the probe set logRatio values of the top10 most up-regulated probe sets lists, the high ZAP70 group had higher logRatios values than the low ZAP70 group for the common probe sets, and these differences were significantly higher.

Finally, the list of the significant probe sets was analyzed with IPA. The top IPA network obtained with the dataset of the probe sets of the interaction term had associated the functions of *cellular death*, *renal necrosis/cell death*, and *liver necrosis / cell death* (Figure 27). Again, it was observed that the genes differently expressed in the two ZAP70 groups were related to apoptosis / cell death.

Moreover, GILZ was one of the top 10 most up-regulated genes by dexamethasone, but only in the high ZAP70 group (see Table 6). Finally, GILZ is a direct target of the GR since its promoter contains six GRE.¹⁶⁵

The combine data suggested that GILZ may be implicated in the different response to dexamethasone observed in the ZAP70 expression groups, consequently GILZ was selected to be studied in a large CLL series.

5. THE EXPRESSION LEVELS OF FKBP5 AND THE RESPONSE TO DEXAMETHASONE

The GEP analyses revealed that FKBP5 was the most inducible gene by dexamethasone in CLL cells. Moreover, they have shown that the levels of FKBP5 were higher both in the untreated cells, and in the treated cells of the high ZAP-70 group. FKBP5 gene codifies for a co-chaperone of the glucocorticoid receptor complex thus it has been hypothesized that the levels of FKBP5 could influence the response of CLL cells to dexamethasone.

5.1. FKBP5 gene expression and the treatment of CLL with dexamethasone for 6 hours

To further analyze the relationship between FKBP5 and the response to dexamethasone in CLL samples, the expression of this gene was ascertained by QRT-PCR. A series of 43 CLL samples was studied; 20 samples had high ZAP70 expression and the remaining 23 had low ZAP70 expression. FKBP5 gene expression was determined in the cells treated with dexamethasone for 6 hours and in the untreated cells, and the induction of FKBP5 (fold change) was determined as the ratio between them. The response to dexamethasone was evaluated after 24 hours of treatment with the drug (Table 7).

Table 7. FKBP5 gene expression in CLL samples treated with dexamethasone for 6 hours

Sample number	ZAP70 category	Live cells (%)	FKBP5 untreated (AU)	FKBP5 treated (AU)	FKBP5 FC
1	low	79		2.51	
2	low	100	0.08	1.17	15.14
3	low	98	0.15	2.16	14.12
4	low	94	0.25	0.91	3.63
5	low	97	0.24	2.22	9.25
6	low	86	0.11	2.84	25.11
7	low	89	0.15	1.1	7.57
8	low	92	0.16	2.24	13.93
9	low	85	0.43	2.56	5.94
10	low	95	0.17	1.19	7.06
11	low	76	0.21	2.55	11.96
12	low	86	0.35	1.57	4.5
13	low	93	0.45	2.72	6.02
14	low	83	0.14	1.22	8.69
17	low	74		1.55	
19	low	82	0.18	2.47	13.7
20	low	73	0.22	2.23	10.2
21	low	100	0.21	1.54	7.52
22	low	86	0.19	2.14	11.39
23	low	59	0.34	2.69	7.84
24	low	91	0.34	1.37	4.08
25	high	51	0.26	3.97	15.14
26	high	37	0.75	10.91	14.62
27	high	33	0.45	4.52	10.13
28	high	81	0.33	2.63	7.94
29	high	77	0.31	3.52	11.65
30	high	61	0.58	6.17	10.63
31	high	44	0.8	7.49	9.32
32	high	43	0.54	2.65	4.96
34	high	36	0.63	3.47	5.5
35	high	69	0.82	6.84	8.34
36	high	60	0.5	3.24	6.54
37	high	80	0.06	0.39	6.82
39	high	77	0.51	3.62	7.16
40	high	76	0.15	2.08	14.12
41	high	68	0.61	2.9	4.79
43	high	63	0.62	7.86	12.64
44	high	70	0.38	5.12	13.64
45	high	67	0.17	1.48	8.82
47	low	95	0.27	2.79	10.48
48	low	80	0.23	1.03	4.5
49	high	84	0.33	2.44	7.46
50	high	69	0.97	4.85	4.99

Live cells (%): percentage of live cells after treatment with dexamethasone relative to the percentage of live cells in the untreated cells; FKBP5 FC: FKBP5 fold change. Sample number according to Table 4.

Results

In accordance with the results of the GEP analyses, the mRNA levels of FKBP5 in the untreated cells were significantly higher in the high ZAP70 group (n=20) than in the low ZAP70 group (n=21) (mean FKBP5 mRNA expression \pm SD; 0.49 AU \pm 0.24 vs 0.23 AU \pm 0.10; $P < 0.001$; Figure 28).

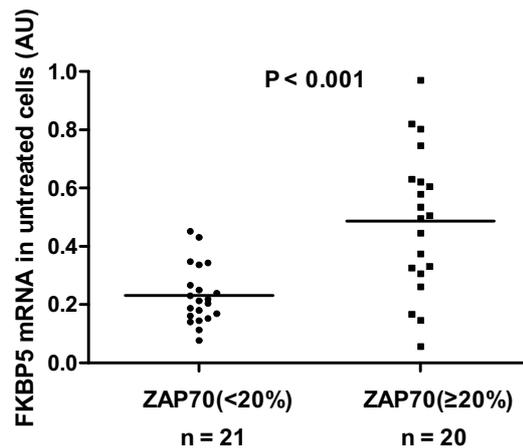


Figure 28. FKBP5 mRNA expression levels in the untreated cells according to ZAP70 groups

CLL cells were treated with 13.25 μ M of dexamethasone for 6 hours, and the levels of FKBP5 mRNA expression were determined by QRT-PCR after 6 hours in the cells left with standard medium. Untreated cells from the high ZAP70 group show higher levels of FKBP5 than cells from the low ZAP70 group. Horizontal bars represent the mean value of FKBP5 mRNA expression (AU).

The FKBP5 expression was highly induced after 6 hours of dexamethasone treatment. FKBP5 was induced 9.46 fold in mean, and did not differ between ZAP70 groups (FKBP5 FC \pm SD: high ZAP70 (n=20) 9.26 ± 3.40 vs low ZAP70 (n=21) 9.65 ± 5.00). In accordance, the levels of FKBP5 mRNA expression (mean FKBP5 mRNA expression \pm SD) were significantly higher in the treated cells (n=43; $3.04 \text{ AU} \pm 2.12$) than in the untreated cells (n=41; $0.36 \text{ AU} \pm 0.22$) $P < 0.001$ (Figure 29).

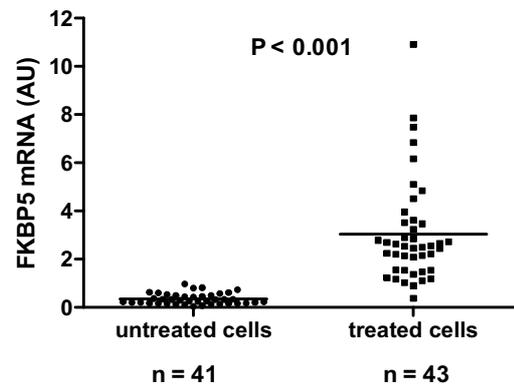


Figure 29. FKBP5 mRNA expression levels in untreated and dexamethasone treated CLL cells

CLL cells were treated with $13.25 \mu\text{M}$ of dexamethasone, and the levels of FKBP5 mRNA expression were determined by QRT-PCR after 6 hours. Treated cells show higher levels of FKBP5 than untreated cells. Horizontal bars represent the mean value of FKBP5 mRNA expression (AU).

Results

The levels of FKBP5 reached after 6 hours of treatment with dexamethasone were significantly higher in the cases with high ZAP70 expression (n=20) than in those with low ZAP70 (n=23) (mean FKBP5 mRNA expression \pm SD: 4.31 AU \pm 2.51 vs 1.95 AU \pm 0.65; P<0.001; Figure 30).

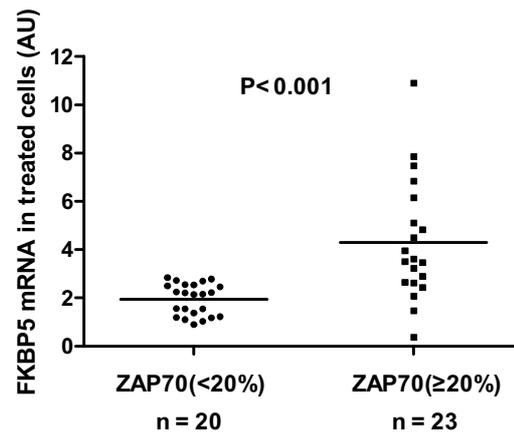


Figure 30. FKBP5 mRNA expression levels in the treated cells according to ZAP70 groups

CLL cells were treated with 13.25 μ M of dexamethasone, and the levels of FKBP5 mRNA expression were determined by QRT-PCR after 6 hours in the treated cells. Treated cells from the high ZAP70 group show higher levels of FKBP5 than cells from the low ZAP70 group. Horizontal bars represent the mean value of FKBP5 mRNA expression (AU).

Since both the expressions of FKBP5 in untreated and in treated cells, were proved to be higher in the high ZAP70 group, the one with the better responses to dexamethasone, it was thought that the gene expression levels of FKBP5 could be related to the magnitude of the response to dexamethasone. Indeed, an inverse correlation between FKBP5 mRNA expression levels in untreated cells and the percentage of live cells was observed ($P < 0.001$; Figure 31A). As well, FKBP5 mRNA expression levels in treated cells were inversely correlated with the percentage of live cells ($P < 0.001$; Figure 31B).

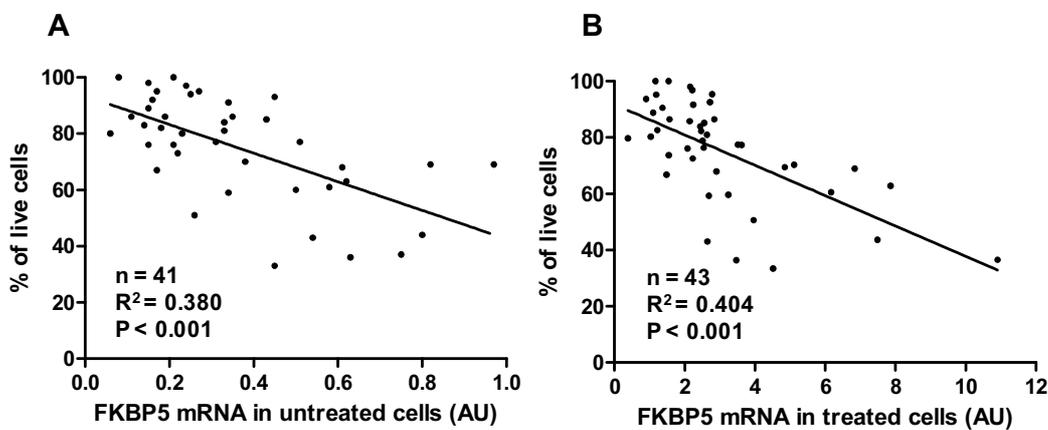


Figure 31. FKBP5 mRNA expression levels in untreated and in treated cells, and their correlation with the response to dexamethasone

CLL cells were treated with 13.25 μM of dexamethasone for 24 hours. Dexamethasone responses were determined at 24 hours. The levels of FKBP5 mRNA expression were determined by QRT-PCR at 6 hours. (A) Scatter-plot showing a significant negative correlation between the percentage of live cells and the mRNA expression levels of FKBP5 in untreated cells. (B) Scatter-plot showing a significant negative correlation between the percentage of live cells and the mRNA expression levels of FKBP5 in treated cells.

5.2. FKBP5 gene and protein levels at baseline

The FKBP5 determinations on untreated cells could present bias due to cell culture, and thus could not reflect baseline features. In order to discard possible bias, FKBP5 gene basal levels were determined by QRT-PCR. Furthermore, the baseline protein levels of FKBP5 were analyzed by immunoblotting. A total of 38 CLL samples were studied, of them, 16 had high ZAP70 expression. The response to dexamethasone was evaluated after 24 hours of treatment (Table 8).

Table 8. FKBP5 gene and protein expressions in CLL cells at baseline

Sample number	ZAP70 category	Live cells (%)	FKBP5 mRNA (AU)	FKBP5 protein (AU)
1	low	79	0.6	
2	low	100	0.25	
3	low	98	0.68	0.164
4	low	94	0.86	
5	low	97	0.93	0.101
6	low	86	0.59	0.119
7	low	96	0.3	
8	low	92	0.37	0.2
9	low	85	0.76	0.127
10	low	95	0.59	0.091
11	low	76	0.43	
12	low	86	0.45	0.325
14	low	83	0.33	0.329
15	low	40	0.76	0.476
17	low	74	0.58	0.276
19	low	82	0.87	0.149
20	low	73	0.76	0.149
22	low	86	0.25	
23	low	59	0.82	0.065
24	low	91	0.38	0.054
26	high	37	1.8	0.153
28	high	81	0.45	0.075
30	high	61	0.72	0.28
31	high	68	1.57	
32	high	43	1.48	0.633
34	high	36	1.04	0.625
35	high	69	1.17	0.844
37	high	80	0.3	
40	high	76	0.44	
41	high	68	0.62	0.633
42	high	96	2.31	
43	high	63	0.39	0.0819
44	high	70		0.635
45	high	67	0.82	0.578
46	high	83	0.75	
47	low	95	0.73	0.373
48	low	80	0.24	0.102
49	high	84	0.48	0.331
50	high	69	0.9	0.443

Live cells (%): percentage of live cells after treatment with dexamethasone relative to the percentage of live cells in the untreated cells. Sample number according to Table 4.

The results of FKBP5 gene expression determined at baseline were equivalent to those obtained in the untreated cells. The levels of FKBP5 were higher in the CLL cases with high ZAP70 expression (n=16) than in the cases with low ZAP70 (n=22) (mean levels of FKBP5 mRNA expression \pm SD: 0.95 AU \pm 0.58 vs 0.57 AU \pm 0.22; P=0.032; Figure 32A). Moreover, FKBP5 baseline levels correlated with the response to treatment with dexamethasone (n=38; P=0.027; Figure 32B).

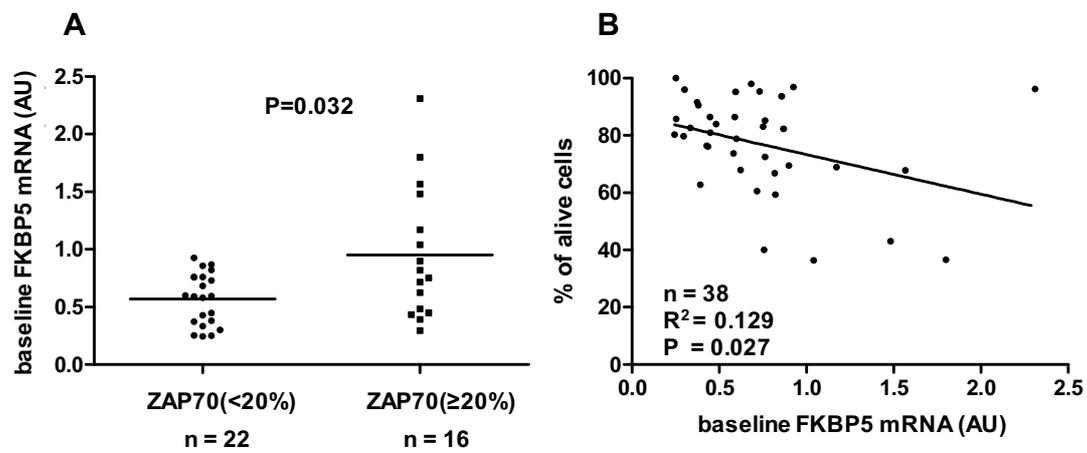


Figure 32. FKBP5 mRNA expression levels at baseline according to ZAP70 groups, and their correlation with the response to dexamethasone

CLL cells were treated with 13.25 μ M of dexamethasone for 24 hours. Dexamethasone responses were determined at 24 hours. The levels of FKBP5 expression were determined by QRT-PCR at baseline. (A) At baseline the cells from the high ZAP70 group show higher levels of FKBP5 than cells from the low ZAP70 group. Horizontal bars represent the mean value of FKBP5 mRNA expression (AU). (B) Scatter-plot showing a significant negative correlation between the percentage of live cells and the mRNA expression levels of FKBP5 mRNA at baseline.

The baseline levels of FKBP5 protein were analyzed in 28 CLL samples by immunoblotting (Figure 33) and subsequently quantified using the Image Gauge V4.0 software (Table 8). The response to dexamethasone was evaluated after 24 hours of treatment (Table 8).

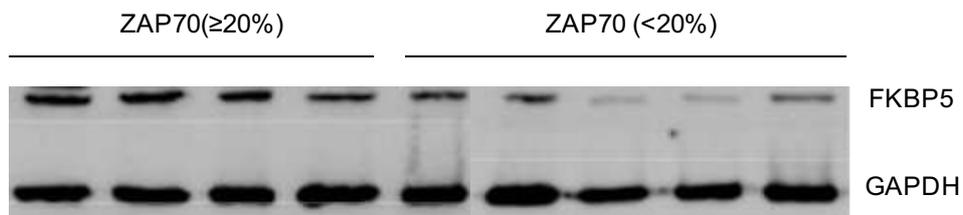


Figure 33. Immunoblotting analyses of FKBP5 protein expressions in CLL cells at baseline

Example of the immunoblotting analyses of FKBP5 protein expression levels at baseline. FKBP5 expression levels were normalized to the expression levels of GAPDH.

The protein levels of FKBP5 were higher in the CLL cases with high ZAP70 expression (n=12) than in the cases with low ZAP70 expression (n=16) (mean levels of FKBP5 protein expression \pm SD: 0.443 AU \pm 0.254 vs 0.194 AU \pm 0.125; P=0.013; Figure 34A). Moreover, FKBP5 protein expressions were inversely correlated with the percentage of live cells (P=0.017; Figure 34B).

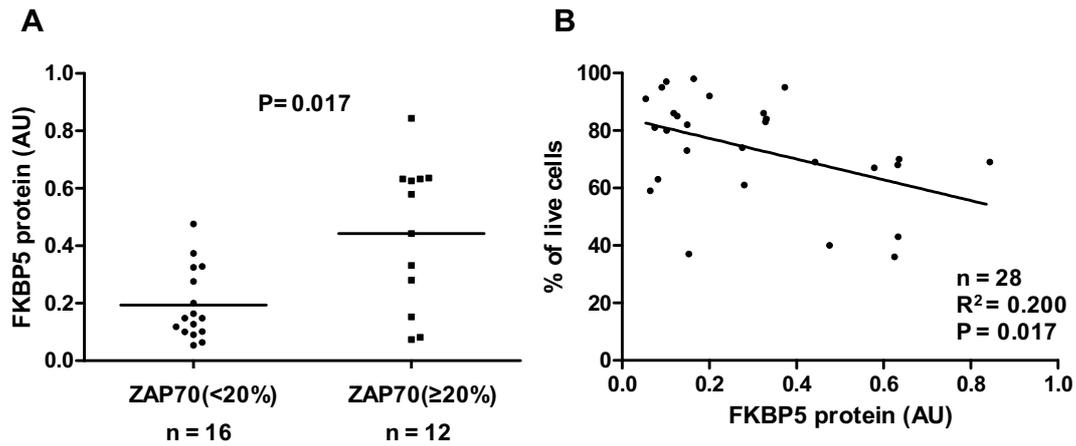


Figure 34. FKBP5 protein expressions according to ZAP70 groups, and their correlation with the response to dexamethasone

CLL cells were treated with 13.25 μ M of dexamethasone for 24 hours. Dexamethasone responses were determined at 24 hours. The protein levels of FKBP5 were analyzed by immunoblotting at baseline. (A) The cells from the high ZAP70 group show higher levels of FKBP5 than the cells from the low ZAP70 group. Horizontal bars represent the mean value of FKBP5 protein expression (AU). (B) Scatter-plot showing a significant negative correlation between the percentage of live cells and the protein levels of FKBP5.

The results obtained for the FKBP5 protein were in line with the obtained for the FKBP5 gene. High levels of this co-chaperone of the GR were correlated with better responses to dexamethasone.

6. RESPONSE TO DEXAMETHASONE AND INDUCTION OF GILZ EXPRESSION

GILZ is a transcription regulator directly targeted by the GR and negatively controls important mediators of cell proliferation.^{165;166} According to GEP analyses, GILZ was one of the top ten most inducible genes, but only in the high ZAP70 group (Table 6). Moreover, GILZ was one of the few genes differently regulated by dexamethasone in the two ZAP70 groups (interaction term; Figure 26).

To further assess the relationship between GILZ expression, ZAP70 expression, and the response to dexamethasone, the levels of GILZ mRNA were determined by QRT-PCR in 40 CLL samples with or without treatment with dexamethasone for 6 hours. The response to dexamethasone was evaluated after 24 hours of treatment (Table 9).

Table 9. GILZ mRNA expression in CLL samples after 6 hours of treatment with dexamethasone

Sample number	ZAP70 category	Live cells (%)	GILZ untreated (AU)	GILZ treated (AU)	GILZ FC
2	low	100	37.77	120.18	3.18
3	low	98	53.78	213.63	3.97
4	low	94	30.46	167.61	5.5
6	low	86	31.1	116.08	3.73
7	low	89	84.98	240.35	2.83
8	low	92	24.89	96.84	3.89
9	low	85	43.94	149.86	3.41
10	low	95	26.86	114.36	4.26
11	low	76	92.99	261.2	2.81
12	low	86	30.68	139.97	4.56
13	low	93	14.5	58.79	4.06
14	low	83	54.53	178.4	3.27
19	low	82	75.01	214.67	2.86
20	low	73	37.47	112.79	3.01
21	low	100	22.75	78.66	3.46
22	low	86	32.88	163.03	4.96
23	low	59	29.84	127.91	4.29
24	low	91	20.95	98.29	4.69
25	high	51	68.55	351.89	5.13
26	high	37	13.54	164.16	12.13
27	high	33	16.42	66.6	4.06
28	high	81	23.74	107.56	4.53
29	high	77	80.86	234.98	3.03
30	high	61	29.19	134.13	4.6
31	high	44	19.39	178.22	9.19
32	high	43	26.89	156.39	5.82
34	high	36	10.76	73.39	6.82
35	high	69	17.24	110.47	6.41
36	high	60	63.96	295.91	4.63
37	high	80	10.19	44.91	4.41
39	high	77	23.25	125.28	5.39
40	high	76	69.02	243.71	3.53
41	high	68	14.41	91.71	6.36
43	high	63	24.89	153.01	6.15
44	high	70	28.99	178.22	6.15
45	high	67	27.65	121.85	4.41
47	low	95	29.43	159.68	5.43
48	low	80	34.27	146.93	4.29
49	high	84	57.64	144.91	2.51
50	high	69	27.65	180.89	6.54

Live cells (%): percentage of live cells after treatment with dexamethasone relative to the percentage of live cells in the untreated cells; GILZ FC: GILZ fold change. Sample number according to Table 4.

In untreated samples, the levels of GILZ were higher in the low ZAP70 group (n=20) than in the high ZAP70 group (n=20) (mean GILZ mRNA expression \pm SD: 40.45 AU \pm 21.46 vs 32.71 AU \pm 22.05; P=0.040; Figure 35A). In all CLL samples, cell treatment with dexamethasone led to the induction of GILZ (range 2.51 to 12.13). Conversely, and according to GEP results, GILZ was significantly more induced in samples with high ZAP70 expression (n=20) than in those with low ZAP70 (n=20) (mean GILZ fold change \pm SD: 5.59 \pm 2.16 vs 3.92 \pm 0.83; P=0.002; Figure 35B).

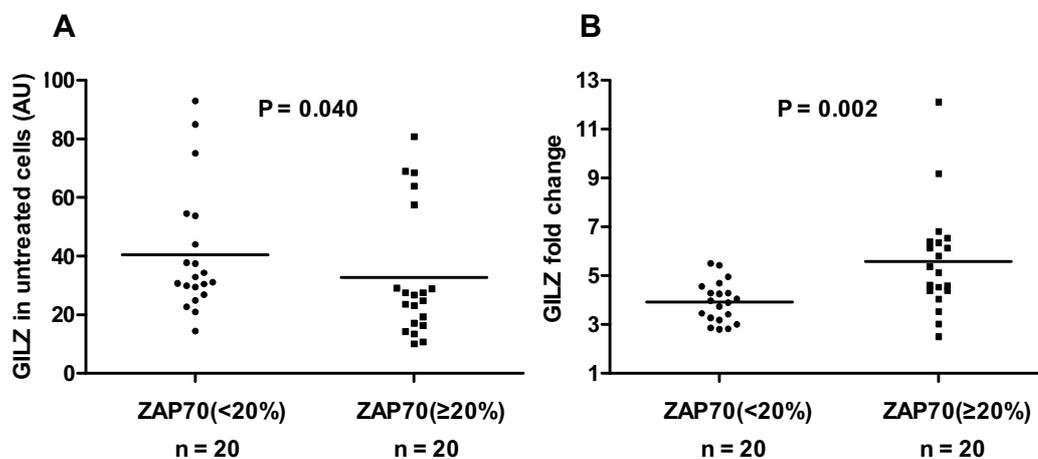


Figure 35. GILZ mRNA expression in untreated CLL cells and GILZ induction after treatment with dexamethasone according to ZAP70 groups

The levels of expression of GILZ were determined by QRT-PCR after 6 hours of treatment. (A) The untreated cells from the cases with low expression of ZAP70 show higher levels of GILZ mRNA than the cells from the cases with high ZAP70. (B) After 6 hours of treatment with dexamethasone, cases with high ZAP70 expression show higher induction of GILZ than cases with low ZAP70. In (A) and (B), horizontal bars represent the mean value of the y-axis units.

Importantly, the induction of GILZ correlated with the response to treatment with dexamethasone (n=40; P<0.001; Figure 36A). The CLL cases with higher levels of GILZ induction at 6 hours of treatment presented higher levels of apoptosis at 24 hours. Moreover, it was observed a correlation between the induction of GILZ at 6 hours, and the induction of the pro-apoptotic BIM at 24 hours (n=34; P=0.001; Figure 36B). This finding reinforced the role of GILZ in the molecular mechanism of dexamethasone cell death and was in line with the clustering of GILZ and BIM in the GEP interaction term analysis.

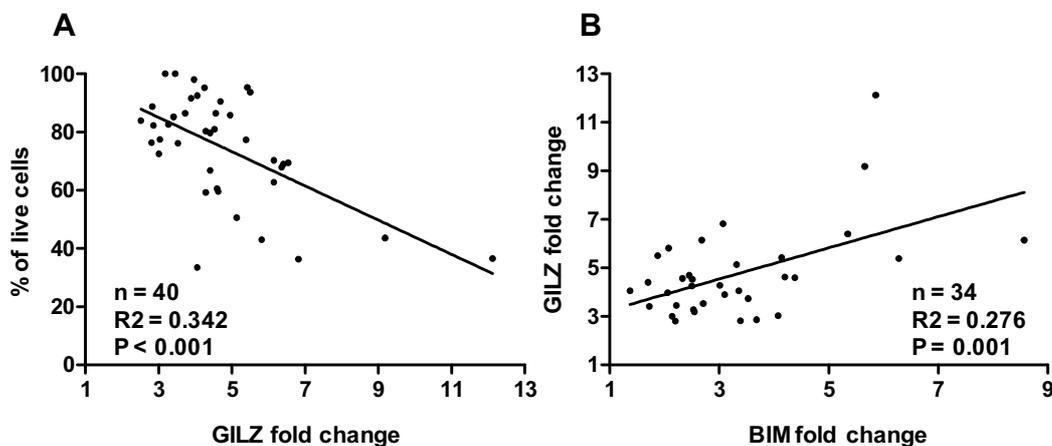


Figure 36. GILZ induction correlations with the response to dexamethasone and BIM induction

CLL cells were treated with 13.25 μ M of dexamethasone for 24 hours. Dexamethasone responses were determined at 24 hours. Fold change of GILZ and BIM expressions were determined by QRT-PCR respectively at 6 and 24 hours. (A) Scatter-plot showing a significant negative correlation between GILZ induction and the percentage of live cells after treatment with dexamethasone. (B) Scatter-plot showing a significant positive correlation between the induction of BIM and GILZ.

DISCUSSION

Discussion

DISCUSSION

Glucocorticoids are part of the therapeutic armamentarium of CLL for a long time. Nowadays, there is a resurgent interest in the use of glucocorticoids in CLL because many of the CLL cells have, or acquire, TP53 abnormalities, and glucocorticoids induce cell death independently of p53.¹⁵⁸ Despite the broad use of glucocorticoids in CLL, there is scarce information regarding the mechanisms by which they induce cell death. This work aims to elucidate the molecular mechanisms behind the apoptosis induced by glucocorticoids in CLL cells, and to identify which groups of patients are prone to benefit more from the use of these drugs.

Recent studies showed that the degree of apoptosis induced by prednisone or by methylprednisolone in CLL cells *ex-vivo* is higher in patients with unmutated IGHV genes and / or high ZAP70 expression than in patients without these adverse prognostic markers.²⁰⁹⁻²¹² This observation was very interesting and made important the study of the response to dexamethasone in CLL cells according to IGHV mutational status and ZAP70 expression. The results from this thesis ascertain that, like as for other glucocorticoids, the response to dexamethasone is significantly higher in the CLL cases with the adverse prognostic markers unmutated IGHV genes and high ZAP70 expression.

The response to dexamethasone was compared between CLL patient groups defined by the presence or absence of high-risk cytogenetics, namely 17p13 and 11q22-q23 deletions. These deletions affect respectively the TP53 and ATM genes, and like unmutated IGHV genes and high ZAP70 expression, both have been shown to have adverse prognostic value.^{70;71} A very small number of patients with CLL in this work series has high-risk genetic abnormalities, and thus the finding that patients with CLL with high-risk cytogenetics have significantly higher responses to dexamethasone than patients without those abnormalities should be confirmed in larger studies. Notwithstanding, the results of this thesis corroborate the clinical experience on the use

of glucocorticoids in patients with high-risk cytogenetics.^{215;218} Furthermore, when analyzing the response to dexamethasone in patients with CLL without high-risk cytogenetics, it is observed that patients with high ZAP70 expression have higher responses to the drug than patients with low ZAP70. It can be concluded that ZAP70 expression has predictive value for the response of CLL cells to dexamethasone, independently of the presence of high-risk cytogenetics.

So far, the biological importance of ZAP70 in the induction of CLL apoptosis has not been uncovered. A recent report has shown that ZAP70 levels are reduced during treatment of CLL cells with methylprednisolone.²¹¹ The authors, although, have found that the inhibition of ZAP70 induction did not influence the response to the drug. Thus, it seems that signals mediated by ZAP70 have no relation with the apoptotic mechanisms induced by the glucocorticoids. The CLL cells with high ZAP70 expression must have other molecular characteristics that justify their different behavior to glucocorticoids.

The induction of BIM expression was shown to be implicated in the apoptosis induced by dexamethasone in ALL,^{161;230-232} and this protein appeared to be the unique pro-apoptotic protein involved in cell death induced by glucocorticoids in CLL cells.²⁰⁴ For these reasons, the expression of BIM mRNA was evaluated in CLL cells after the treatment with dexamethasone. The results of this thesis show that BIM is induced in all CLL cases after 24 hours of dexamethasone treatment, this correlating with the response to the drug. However, some cases do not respond to dexamethasone induced cell death, indicating either that additional pathways are involved in induction of apoptosis in these cells, or that the response to the treatment is delayed beyond the observed 24 hours. In addition, CLL cases with high ZAP70 expression, which are the best responders to dexamethasone, show significantly higher induction of BIM than cases with low ZAP70 expression. This observation suggests that the molecular mechanisms behind the different response to dexamethasone observed between CLL

groups defined by ZAP70 expression may reside upstream the pro-apoptotic BIM. Finally, since the pro-apoptotic mechanism of BIM has been demonstrated to be independent from p53,¹⁵⁸ the up-regulation of BIM could explain in part, the response to glucocorticoids observed in the CLL cases with TP53 abnormalities.

BIM expression is early induced after the treatment with dexamethasone, achieving the maximum peak after 9 hours of treatment in most of the cases. As a consequence, the time point at 6 hours after treatment was chosen to study the genes regulated by dexamethasone that could explain the different responses to this drug.

The comparison of gene expression profiling of CLL cells with high or low ZAP70 expression treated with dexamethasone reveals several interesting differences. Unsupervised analysis of the genes with the highest variation in expression, defined two main groups according to ZAP-70 expression, clustering together samples from the same patient with and without treatment. Of note, previous studies of GEP of CLL cells were not able to discriminate, by means of unsupervised analysis, the cases with unmutated IGHV genes / high ZAP70 expression from those with mutated IGHV genes / low ZAP70, and subsequent supervised analysis supported that CLL cells has quite a homogenous phenotype.^{32;66;234-237} This indicates that treatment with dexamethasone is able to induce enough changes in gene expression as to separate CLL cases according to ZAP70 expression.

The following steps of the analysis of the GEPs data were the identification and comparison of the genes induced and repressed by dexamethasone in the CLL groups defined by ZAP70 expression. The two CLL groups were studied separately, and by means of supervised analysis, the probe set lists with the genes up and down-regulated by dexamethasone were obtained. Further, different analysis approaches were performed in order to identify the differences in gene regulation between the two groups. First, by means of GO analyses, the biological processes overrepresented in the lists of probe sets generated were ascertained. In both ZAP70 groups, the induction

of gene expression plays a more relevant role in the apoptosis than the repression since, in general, the biological processes overrepresented in the up-regulated probe set lists are related to apoptosis, whereas in the down-regulated probe set lists, they are related to immune response. Moreover, the analyses of the common and uncommon probe sets up-regulated in ZAP70 expression groups show that the list of probe sets solely induced in the cells belonging to the high ZAP70 group has an enrichment in genes related to apoptosis, which is in line with the higher apoptotic effects observed in this group. Additionally, the low ZAP70 group solely down-regulated probe set lists is enriched in genes related to the regulation of apoptosis, and the detailed analysis of these probe sets shows that they recognize genes inducers of apoptosis. The down-regulation of apoptosis inducing genes in the low ZAP70 expression cases could in part explain the lower response to dexamethasone induced cell death observed in this group.

The second analysis approach to identify the differences in gene regulation between ZAP70 expression groups was the comparison of the top 10 probe sets with the highest variation between groups. It was observed that the top 10 probe set lists of the two CLL groups have many probe sets in common. Although, the degree of induction / repression of the common probe sets is higher in the high ZAP70 expression group, which is the CLL group with the superior responses to dexamethasone.

The third analysis approach was the analysis of the probe set list containing the up and down-regulated genes in the high ZAP70 group with the IPA tool. The top IPA network includes many of the top 10 most up and down-regulated genes and has associated the function of *cellular growth and proliferation, hematological system development, and function and tissue development*. Apparently, dexamethasone treatment interferes with cell growth and proliferation processes which can contribute to the induction of apoptosis. IPA tool was also used to highlight the genes regulated by

dexamethasone that are part of the *canonical pathway of the glucocorticoid receptor signaling* like FKBP5.

Supervised analysis was conducted to retrieve the genes differently regulated in the ZAP70 expression groups; the resulting probe set list includes few genes, and both GO and IPA analysis show an overrepresentation in genes related to apoptosis. Apparently, there are no other relevant biological processes behind the different response to dexamethasone observed between ZAP70 groups. Among this list of the differently regulated probe sets, special attention has been given to GILZ since it is present in the top IPA network, and its pattern of modulation by dexamethasone is similar to the observed for BIM.

In summary, GEP analyses reveal high similarities between ZAP70 groups in terms of genes regulated by dexamethasone, and indicate that the different response to dexamethasone may be due to a differential capacity to induce cell death while inducing / repressing the same genes.

GEP analyses results allowed the selection of genes with significant levels of modulation along with biological relevance in the glucocorticoid pathway for further studies in a larger series of patients. The most inducible gene after dexamethasone treatment in both ZAP70 groups is FKBP5, a gene that codifies for a co-chaperone of the glucocorticoid receptor.²³⁸ Moreover, GEP analyses show that FKBP5 expression is higher in patients with high ZAP70 expression than in patients with low ZAP70, and these both in the untreated cells and in the dexamethasone treated cells. Analysis of FKBP5 levels in a larger series of samples from patients with CLL demonstrated that the baseline levels of mRNA and protein of FKBP5 correlate with the extent of cell death, being FKBP5 levels higher in the cases with high ZAP70 expression.

The results of this thesis are in line with a previous report in MM cell lines where a correlation between higher initial levels of FKBP5 and the response to dexamethasone in terms of apoptosis, has been observed.²³⁹ Likewise, the levels of

GR have been correlated with the degree of induced apoptosis in a study performed in ALL.²⁴⁰ The importance of FKBP5 has been disclosed by the model for hormonal activation of the GR.^{111;233} This model puts forward that the GR is bound to FKBP5 in the absence of glucocorticoids, and that the binding of the hormone causes the switch of FKBP5 by FKBP4. FKBP4 unlike FKBP5 interacts with dynein thus allowing the translocation of the glucocorticoid-GR complex to the nucleus. The interchange between FKBP5 and FKBP4 is affected by the levels of both co-chaperones particularly by the ratio between them. Interestingly, in some cellular systems, like those using New World primates and squirrel monkey cells, an over-expression of FKBP5 has been related with a reduced transcriptional activity of the GR.^{241;242} The reported inhibitory action of FKBP5 can be explained by the ratio of FKBP5 / FKBP4 observed in those primates; it was 26 fold higher than the ratio observed in humans thus the substitution of FKBP5 by FKBP4 after the glucocorticoid binding would be compromised, and as a consequence, the glucocorticoid-GR complex translocation to the nucleus also. The GEP results of this thesis are indicative that, in CLL cells, the glucocorticoid-GR complexes moves to the nucleus since they are observed GR genomic effects like induction / repression of the transcription of several genes. Moreover, GEP results show that the cells of the cases with high ZAP70 expression have increased levels of up and down-regulation of gene expression, with respect to the cells of the cases with low ZAP70. The higher levels of FKBP5 observed in the CLL cases with high ZAP70 expression can be in part responsible for an increased signaling through the GR and thus for the better response to dexamethasone observed in high ZAP70 cases.

It cannot be ruled out that the higher responses to dexamethasone observed in the CLL cases with higher FKBP5 levels are also due to non-genomic effects like those mediated by the direct interaction of the glucocorticoid-GR complex with cytoplasmatic proteins. It has been reported that glucocorticoid-GR complex effects are not confined to the nucleus and that glucocorticoids are able to impair the phosphorylation of proteins like AKT and MAPK.¹²⁷ The PI3K-AKT signaling pathway is constitutively

active in some human cancers including in CLL, and it promotes cellular survival and resistance to chemotherapy.^{187;243;244} Activated AKT is able to inhibit apoptosis by phosphorylation and subsequent inactivation of pro apoptotic proteins like BAD and caspase 9.^{245;246} Thus in the cytoplasm, and before the replacement of FKBP5 by FKBP4, the glucocorticoid-GR complex could be mediating apoptotic signals through the inactivation of AKT.

GILZ is among the few genes identified in GEP analyses as differently regulated by dexamethasone in the ZAP70 expression groups. In continuation, GEP results were validated by QRT-PCR in a large CLL series; the GILZ induction is significantly higher in the CLL samples with high ZAP70 expression than in those with low ZAP70 expression. Moreover, these thesis results showed that the induction of GILZ correlates with the apoptotic levels induced by the treatment with dexamethasone, and the induction of the downstream apoptotic effector BIM.

Six GRE in the promoter of GILZ have been identified,¹⁶⁵ thus GILZ transcription can be directly regulated by the GR. GILZ has been previously reported to be induced by glucocorticoids in other related cellular systems like ALL and MM.^{164;247;248} Importantly, GILZ has been implicated in cell death after glucocorticoid treatment since its inhibition by siRNA impairs the apoptotic response in MM.²⁴⁸ So far, it has not been described a direct role for GILZ in the apoptotic pathway, however several pieces of evidence support that GILZ can induce cell apoptosis through the modulation of cell survival and cell proliferation pathways. Firstly, GILZ has been shown to associate with RAS and RAF reducing the activation of downstream RAS targets like ERK, AKT, and CCND1.¹⁶⁶ Moreover, GILZ has been shown to inhibit the NFkB and the AP1 transcription factors.^{167;168}

Activated AKT has been shown to inhibit cell death pathways by directly phosphorylation and consequent inactivation of pro apoptotic proteins like BAD and caspase 9.^{245;246} In addition, activated AKT has been reported to increase the activity of

IKK, which leads to the degradation of NF κ B inhibitors such as I κ B α .²⁴⁹ The degradation of I κ B α results in the release of NF κ B, from the cytoplasm to the nucleus, where it acts as a transcription factor. NF κ B has been shown to promote cell survival and to inhibit apoptosis by inducing the expression of the apoptotic inhibitors IAPs, BCLXL, and BCL2A1.^{250;251} CLL cells have been shown to have high constitutive levels of AKT and NF κ B activity, which are dependent of PI3K, and have been implied in the survival of CLL.^{244;252} The results of this thesis point toward a role of GILZ in the apoptosis induced by glucocorticoids in CLL, most likely by down-regulating cell survival and cell proliferation pathways like PI3K / AKT / mTOR and RAS / RAF / MEK / ERK. The higher response to dexamethasone observed in cases with high ZAP70 expression is probably attributable to an increased inhibition of survival and proliferation signals in cells of these cases.

In summary, this thesis provide the first 'gene / molecular fingerprint' of dexamethasone in CLL cells. These thesis results underscore the better responses to glucocorticoids of the CLL cells of patients from the poor outcome group with unmutated IGHV genes / high ZAP70 expression, and describe some genes associated to this differential response. In addition, these results can facilitate the development of predictive markers of response to dexamethasone, since the higher response observed in cases with UCLL / high ZAP-70 expression correlates with the baseline expression of FKBP5, a gene involved in the glucocorticoid pathway. Finally, among the genes regulated by dexamethasone, the identification of GILZ, a gene responsible for the inhibition of pathways like PI3K / AKT / mTOR and RAS / RAF / MEK / ERK contributes to highlight the importance of these cell survival and cell proliferation pathways in CLL cells.

CONCLUSIONS

Conclusions

CONCLUSIONS

1. CLL cases with unmutated IGHV genes / high ZAP70 expression show higher induced apoptosis by dexamethasone than cases with mutated IGHV genes / low ZAP70 expression.
2. CLL cases with high risk cytogenetic features like deletions in 17p13 and 11q22-q23 show high levels of apoptosis induced by dexamethasone.
3. The magnitude of the apoptosis induced by dexamethasone correlates with the induction of BIM, having CLL cases with high ZAP70 expression the highest levels of BIM induction.
4. The treatment of CLL cells with dexamethasone induces changes in the expression of many genes functionally related with apoptosis, cell survival and proliferation.
5. The different levels of apoptosis induced by dexamethasone observed in the CLL groups defined by ZAP70 expression translate into different profiles of gene expression. These differences are mainly quantitative; cases with high ZAP70 expression show higher levels of gene induction / repression than cases with low ZAP70 expression.
6. Baseline mRNA and protein expression levels of FKBP5, the co-chaperone of the glucocorticoid receptor, correlate with the extent of CLL cells apoptosis induced by the treatment with dexamethasone. Baseline FKBP5 levels are higher in samples from patients with high ZAP70 expression.

Conclusions

7. GILZ is differently induced by dexamethasone in ZAP70 expression groups of CLL, being higher in cases with high ZAP70 expression. Induction of GILZ correlates with induction of BIM and with the levels of apoptosis.

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APPENDIXES

APPENDIX 1

1.1. Probe sets up-regulated by dexamethasone in the high ZAP70 group

Probe set	Gene symbol	LogRatio	FDR
224856_at	FKBP5	3.2471	3.49E-07
202887_s_at	DDIT4	3.1364	1.15E-07
204560_at	FKBP5	3.0735	7.45E-06
218113_at	TMEM2	2.9422	3.22E-08
207001_x_at	TSC22D3	2.7904	9.49E-08
224840_at	FKBP5	2.6932	1.04E-08
226625_at	TGFBR3	2.5250	9.77E-06
204731_at	TGFBR3	2.4958	1.24E-04
242551_at	C18orf1	2.3835	7.73E-09
242406_at	---	2.3142	1.74E-08
203221_at	TLE1	2.2437	1.60E-07
215528_at	---	2.1606	1.04E-08
238423_at	SYTL3	2.1119	1.20E-07
218346_s_at	SESN1	2.1059	1.28E-05
222281_s_at	---	2.1050	1.15E-07
1555372_at	BCL2L11	2.0714	1.25E-09
1562028_at	CCND3	2.0322	5.02E-09
1558143_a_at	BCL2L11	2.0129	3.04E-09
241893_at	---	1.9170	1.54E-05
208763_s_at	TSC22D3	1.8761	6.63E-08
225116_at	HIPK2	1.8409	7.64E-06
224839_s_at	GPT2	1.8373	6.64E-08
230381_at	C1orf186	1.8352	6.64E-08
219888_at	SPAG4	1.8256	5.52E-08
225606_at	BCL2L11	1.8085	3.04E-09
207651_at	GPR171	1.7556	7.07E-08
214238_at	---	1.7394	4.94E-07
219911_s_at	SLCO4A1	1.7106	1.96E-07
204618_s_at	GABPB1	1.6918	1.04E-08
221757_at	PIK3IP1	1.6875	3.96E-08
201700_at	CCND3	1.6849	1.95E-09
201367_s_at	ZFP36L2	1.6791	2.54E-04
203528_at	SEMA4D	1.6666	1.95E-09
212838_at	DNMBP	1.6665	7.95E-08
219753_at	STAG3	1.6661	2.43E-05
206173_x_at	GABPB1	1.6647	7.46E-08
221756_at	PIK3IP1	1.6507	6.63E-08
225767_at	---	1.6495	1.74E-04
228891_at	SEMA4D	1.6457	3.04E-09
212098_at	LOC151162 /// MGAT5	1.6380	1.59E-06
222334_at	---	1.6310	5.03E-07
205798_at	IL7R	1.6113	1.86E-07
226218_at	IL7R	1.6093	1.19E-07
231979_at	---	1.6088	3.04E-09
210785_s_at	C1orf38	1.6039	6.13E-06
225010_at	CCDC6	1.5902	1.33E-08
1568983_a_at	---	1.5891	2.69E-09

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Probe set	Gene symbol	LogRatio	FDR
219812_at	PVRIG	1.5752	2.22E-07
203542_s_at	KLF9	1.5733	5.42E-07
229822_at	---	1.5670	2.52E-06
37966_at	PARVB	1.5592	6.90E-07
228759_at	CREB3L2	1.5563	1.74E-07
207571_x_at	C1orf38	1.5464	9.09E-07
225115_at	HIPK2	1.5427	2.81E-05
203543_s_at	KLF9	1.5365	5.65E-07
210117_at	SPAG1	1.5338	1.87E-07
242514_at	---	1.5323	1.16E-05
225368_at	HIPK2	1.5315	3.22E-06
244357_at	---	1.5235	4.01E-06
230599_at	---	1.5218	7.10E-09
223085_at	RNF19A	1.5075	3.12E-09
1562208_a_at	---	1.5011	6.20E-03
225097_at	HIPK2	1.4752	2.71E-05
202239_at	PARP4	1.4733	2.49E-08
201369_s_at	ZFP36L2	1.4683	3.69E-05
227406_at	LOC100129387	1.4650	7.83E-07
212345_s_at	CREB3L2	1.4642	5.52E-08
212543_at	AIM1	1.4497	1.64E-05
1565701_at	---	1.4421	6.75E-05
237367_x_at	CFLAR	1.4405	6.64E-08
201161_s_at	CSDA	1.4400	6.13E-06
216834_at	RGS1	1.4296	3.64E-07
201041_s_at	DUSP1	1.4154	1.02E-06
201009_s_at	TXNIP	1.4134	4.67E-05
239328_at	---	1.4115	7.07E-08
1561733_at	---	1.3890	2.71E-05
208485_x_at	CFLAR	1.3889	9.74E-09
211862_x_at	CFLAR	1.3833	1.04E-08
37965_at	PARVB	1.3726	3.22E-06
227312_at	SNTB2	1.3658	6.90E-07
211317_s_at	CFLAR	1.3591	7.10E-09
238393_at	---	1.3584	2.69E-05
204716_at	CCDC6	1.3532	1.25E-06
242527_at	---	1.3429	2.88E-05
220285_at	FAM108B1	1.3258	9.60E-05
229670_at	---	1.3152	6.36E-07
209508_x_at	CFLAR	1.2930	2.06E-08
225762_x_at	LOC284801	1.2874	2.62E-04
220483_s_at	RNF19A	1.2857	3.17E-06
216253_s_at	PARVB	1.2818	2.88E-05
235213_at	ITPKB	1.2769	2.23E-05
203110_at	PTK2B	1.2750	2.04E-06
231296_at	---	1.2642	7.17E-06
243757_at	---	1.2543	1.25E-07
201008_s_at	TXNIP	1.2539	8.01E-05
210564_x_at	CFLAR	1.2516	3.22E-08
230689_at	---	1.2454	1.44E-05
203521_s_at	ZNF318	1.2452	8.82E-06
232722_at	RNASET2	1.2426	1.24E-05
1568943_at	INPP5D	1.2423	2.64E-04

Probe set	Gene symbol	LogRatio	FDR
231873_at	BMPR2	1.2262	1.94E-06
225763_at	RCSD1	1.2260	3.81E-05
219028_at	HIPK2	1.2245	3.90E-03
202962_at	KIF13B	1.2086	2.68E-05
242946_at	---	1.2063	3.05E-06
235427_at	---	1.1991	1.62E-07
227551_at	FAM108B1	1.1981	1.33E-08
223028_s_at	SNX9	1.1957	1.20E-06
203331_s_at	INPP5D	1.1944	1.73E-04
1553096_s_at	BCL2L11	1.1918	1.82E-06
204995_at	CDK5R1	1.1916	2.18E-03
240260_at	---	1.1881	1.17E-05
205882_x_at	ADD3	1.1847	5.65E-07
210563_x_at	CFLAR	1.1840	6.64E-08
218935_at	EHD3	1.1799	1.31E-03
232583_at	---	1.1788	2.74E-06
223027_at	SNX9	1.1708	8.29E-06
50221_at	TFEB	1.1682	9.93E-07
235274_at	---	1.1562	6.90E-07
203085_s_at	TGFB1	1.1561	2.05E-06
227510_x_at	MALAT1	1.1515	9.17E-04
1562600_at	---	1.1452	1.60E-05
232784_at	---	1.1446	1.46E-05
218638_s_at	SPON2	1.1435	1.84E-05
241435_at	---	1.1429	2.01E-06
201752_s_at	ADD3	1.1378	7.47E-07
228308_at	FKBP11	1.1354	7.07E-05
225827_at	EIF2C2	1.1345	7.18E-05
1557557_at	LOC100129196	1.1236	2.30E-05
204546_at	KIAA0513	1.1207	1.09E-04
AFFX-M27830_M_at	---	1.1200	1.01E-04
210214_s_at	BMPR2	1.1193	2.22E-07
203574_at	NFIL3	1.1155	9.65E-05
203006_at	INPP5A	1.1149	3.33E-06
1557673_at	---	1.1148	2.54E-04
236458_at	---	1.1144	3.29E-06
225144_at	BMPR2	1.1117	1.80E-06
232623_at	LOC100128751	1.1104	1.06E-04
1562265_at	---	1.1010	4.59E-05
226685_at	SNTB2	1.0962	6.18E-05
219371_s_at	KLF2	1.0950	5.56E-05
226810_at	OGFRL1	1.0929	4.40E-04
1568997_at	---	1.0911	1.54E-05
243546_at	---	1.0856	7.26E-04
240410_at	---	1.0818	1.13E-03
203332_s_at	INPP5D	1.0798	3.03E-04
243509_at	---	1.0763	3.59E-04
214486_x_at	CFLAR	1.0756	2.22E-07
205315_s_at	SNTB2	1.0746	4.59E-05
232007_at	AGPAT5	1.0689	2.91E-04
239388_at	---	1.0685	5.42E-07
225164_s_at	EIF2AK4	1.0654	6.13E-06
213370_s_at	SFMBT1	1.0650	4.12E-07

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Probe set	Gene symbol	LogRatio	FDR
200681_at	GLO1	1.0640	6.64E-08
202716_at	PTPN1	1.0582	2.27E-05
229540_at	RBPJ	1.0570	1.72E-04
215147_at	---	1.0554	2.85E-06
238071_at	LCN10 /// LCN6	1.0543	5.53E-05
236341_at	CTLA4	1.0491	2.56E-04
242842_at	---	1.0483	1.24E-03
240665_at	---	1.0467	5.05E-05
208190_s_at	LSR	1.0404	7.61E-04
217787_s_at	GALNT2	1.0399	6.89E-06
203520_s_at	ZNF318	1.0390	1.86E-04
210606_x_at	KLRD1	1.0390	6.90E-07
230536_at	PBX4	1.0366	2.63E-04
213622_at	COL9A2	1.0259	2.52E-06
207785_s_at	RBPJ	1.0206	7.86E-04
242492_at	---	1.0200	2.44E-06
239054_at	SFMBT1	1.0122	6.36E-07
205510_s_at	FLJ10038	1.0117	1.04E-04
244592_at	---	1.0048	6.66E-06
204484_at	PIK3C2B	1.0025	1.27E-05
226002_at	GAB1	1.0013	6.51E-05
205002_at	AHDC1	0.9966	1.78E-03
1554569_a_at	CELF2	0.9951	3.24E-02
1555355_a_at	ETS1	0.9940	3.35E-04
209678_s_at	PRKCI	0.9845	1.50E-04
209574_s_at	C18orf1	0.9818	1.72E-05
230740_at	EHD3	0.9783	4.40E-04
234362_s_at	CTLA4	0.9749	2.39E-03
232864_s_at	AFF4	0.9723	5.99E-07
1559739_at	CHPT1	0.9705	7.61E-04
241613_at	---	0.9700	3.25E-04
201753_s_at	ADD3	0.9684	6.42E-05
203111_s_at	PTK2B	0.9675	1.01E-04
219118_at	FKBP11	0.9675	1.24E-03
244026_at	---	0.9653	1.06E-03
202745_at	USP8	0.9639	6.58E-06
221866_at	TFEB	0.9596	1.31E-05
225282_at	SMAP2	0.9548	1.47E-05
234151_at	---	0.9538	9.29E-05
240690_at	---	0.9507	1.25E-03
232865_at	AFF4	0.9503	5.09E-06
226221_at	KIAA1432	0.9501	1.27E-05
213174_at	TTC9	0.9457	7.71E-05
209939_x_at	CFLAR	0.9452	2.57E-07
235683_at	SESN3	0.9439	2.37E-04
224681_at	GNA12	0.9414	6.31E-06
211974_x_at	RBPJ	0.9412	2.44E-06
236164_at	FLJ10038	0.9372	5.92E-05
204857_at	MAD1L1	0.9368	1.64E-04
1557558_s_at	LOC100129196	0.9349	6.13E-06
211458_s_at	GABARAPL1 /// GABARAPL3	0.9346	8.51E-06
244358_at	---	0.9343	1.31E-05
229501_s_at	USP8	0.9323	1.13E-06

Probe set	Gene symbol	LogRatio	FDR
204524_at	PDPK1	0.9311	1.58E-04
214405_at	---	0.9300	1.08E-03
209681_at	SLC19A2	0.9272	2.07E-02
242268_at	CELF2	0.9264	4.62E-04
239778_x_at	---	0.9219	1.36E-03
203315_at	NCK2	0.9218	2.26E-05
231109_at	---	0.9180	1.64E-05
225562_at	RASA3	0.9133	7.24E-05
235668_at	PRDM1	0.9114	5.02E-06
244220_at	---	0.9110	3.39E-05
217788_s_at	GALNT2	0.9102	6.15E-06
210786_s_at	FLI1	0.9092	4.92E-03
229050_s_at	SNHG7	0.9091	3.52E-06
212400_at	FAM102A	0.9083	4.37E-04
1564248_at	---	0.9071	1.24E-03
221725_at	WASF2	0.9051	9.30E-05
213763_at	HIPK2	0.9019	8.85E-04
215144_at	---	0.9014	1.95E-04
229114_at	GAB1	0.9003	4.75E-05
219117_s_at	FKBP11	0.8931	1.25E-03
210655_s_at	FOXO3 /// FOXO3B	0.8917	2.14E-03
201642_at	IFNGR2	0.8880	2.95E-04
211316_x_at	CFLAR	0.8869	1.07E-07
201044_x_at	DUSP1	0.8864	8.22E-03
242109_at	SYTL3	0.8838	1.84E-03
224577_at	ERGIC1	0.8832	1.89E-04
244429_at	---	0.8831	4.42E-05
204132_s_at	FOXO3 /// FOXO3B	0.8827	2.00E-03
1557555_at	---	0.8812	5.18E-04
233921_s_at	---	0.8797	7.10E-04
221331_x_at	CTLA4	0.8750	1.87E-03
231794_at	CTLA4	0.8695	1.59E-04
202158_s_at	CELF2	0.8648	2.84E-04
201010_s_at	TXNIP	0.8645	1.66E-04
228153_at	RNF144B	0.8619	5.85E-05
244646_at	---	0.8607	1.07E-04
214439_x_at	BIN1	0.8602	1.89E-04
239171_at	---	0.8602	2.67E-03
240538_at	---	0.8585	7.97E-04
1554676_at	SRGN	0.8575	1.05E-03
239930_at	GALNT2	0.8560	7.26E-06
242320_at	---	0.8530	5.10E-04
221718_s_at	AKAP13	0.8496	2.52E-06
201485_s_at	RCN2	0.8456	8.17E-05
217983_s_at	RNASET2	0.8428	6.69E-06
217643_x_at	---	0.8419	3.66E-04
227222_at	FBXO10	0.8414	7.36E-05
202931_x_at	BIN1	0.8410	1.15E-04
209920_at	BMPR2	0.8409	5.48E-06
1560386_at	---	0.8392	8.82E-04
224261_at	---	0.8361	1.24E-05
217984_at	RNASET2	0.8350	1.03E-05
210201_x_at	BIN1	0.8342	1.39E-04

Appendixes

Probe set	Gene symbol	LogRatio	FDR
226982_at	ELL2	0.8330	8.27E-04
230970_at	---	0.8314	1.36E-03
227062_at	---	0.8314	2.69E-04
223991_s_at	GALNT2 /// LOC100132910	0.8258	8.17E-05
225998_at	GAB1	0.8244	7.55E-05
224563_at	WASF2	0.8229	1.41E-04
201200_at	CREG1	0.8213	1.65E-03
207734_at	LAX1	0.8190	2.96E-04
232542_at	COL9A2	0.8176	6.22E-06
227146_at	QSOX2	0.8154	6.36E-07
219574_at	MARCH1	0.8104	7.79E-05
210202_s_at	BIN1	0.8048	1.23E-03
227611_at	TARSL2	0.8025	3.28E-06
207996_s_at	C18orf1	0.8016	9.69E-05
227178_at	CELF2	0.8014	1.38E-04
239232_at	MSI2	0.7985	1.30E-03
224562_at	WASF2	0.7983	3.78E-04
244453_at	ANKRD53	0.7972	3.55E-04
1562144_at	---	0.7969	2.96E-04
215925_s_at	CD72	0.7956	3.88E-04
218997_at	POLR1E	0.7942	3.39E-05
221790_s_at	LDLRAP1	0.7940	7.45E-06
202156_s_at	CELF2	0.7933	3.18E-04
227999_at	PWWP2B	0.7907	4.22E-03
201160_s_at	CSDA	0.7906	1.34E-06
208869_s_at	GABARAPL1	0.7887	3.88E-04
1560443_at	---	0.7883	5.81E-05
218971_s_at	WDR91	0.7863	3.35E-04
201034_at	ADD3	0.7858	3.64E-05
232213_at	PEL1	0.7858	8.47E-04
225701_at	AKNA	0.7855	4.32E-05
226099_at	ELL2	0.7804	1.09E-04
224576_at	ERGIC1	0.7784	3.72E-03
225154_at	SYAP1	0.7762	8.33E-05
219256_s_at	SH3TC1	0.7761	1.11E-03
1570165_at	---	0.7742	2.25E-05
203921_at	CHST2	0.7739	2.50E-04
225033_at	ST3GAL1	0.7707	3.87E-03
221737_at	GNA12	0.7702	4.43E-04
203206_at	FAM53B	0.7701	1.55E-05
204924_at	TLR2	0.7694	7.74E-03
1555831_s_at	LRRC41	0.7693	1.15E-02
1558747_at	SMCHD1	0.7686	2.25E-04
229981_at	SNX5	0.7648	9.69E-05
237018_at	---	0.7643	5.86E-03
202967_at	GSTA4	0.7640	4.90E-04
1555392_at	LOC100128868	0.7610	2.30E-03
230526_at	LOC100131096	0.7608	4.17E-05
AFFX-M27830_5_at	---	0.7604	8.81E-03
223803_s_at	ZCCHC10	0.7546	3.98E-05
239629_at	CFLAR	0.7545	3.81E-05
229310_at	KLHL29	0.7542	2.28E-04
212311_at	SEL1L3	0.7515	6.24E-04

Probe set	Gene symbol	LogRatio	FDR
223836_at	FGFBP2	0.7511	2.10E-03
205119_s_at	FPR1	0.7505	3.19E-03

1.2. Probe sets up-regulated by dexamethasone in the low ZAP70 group

Probe set	Gene symbol	LogRatio	FDR
224840_at	FKBP5	2.9170	9.51E-08
215925_s_at	CD72	2.6706	7.55E-08
224856_at	FKBP5	2.6640	3.77E-05
218113_at	TMEM2	2.4896	3.20E-06
204560_at	FKBP5	2.3253	9.84E-04
212838_at	DNMBP	2.2293	9.13E-08
225116_at	HIPK2	2.0960	3.10E-05
215528_at	---	2.0765	3.31E-07
202887_s_at	DDIT4	2.0048	1.30E-04
241893_at	---	1.9640	1.37E-04
1565701_at	---	1.9401	5.25E-05
210117_at	SPAG1	1.9361	3.31E-07
225115_at	HIPK2	1.8958	5.13E-05
218346_s_at	SESN1	1.8916	3.67E-04
203221_at	TLE1	1.8722	1.58E-05
221756_at	PIK3IP1	1.8420	3.53E-07
205798_at	IL7R	1.8074	9.74E-07
222281_s_at	---	1.7985	9.87E-06
1562028_at	CCND3	1.7855	3.31E-07
225097_at	HIPK2	1.7771	5.78E-05
207651_at	GPR171	1.7627	1.21E-06
238423_at	SYTL3	1.7625	1.26E-05
212098_at	LOC151162 /// MGAT5	1.7584	1.09E-05
241435_at	---	1.7551	3.75E-07
210785_s_at	C1orf38	1.7403	3.70E-05
207001_x_at	TSC22D3	1.7373	1.39E-04
218935_at	EHD3	1.7347	5.83E-04
221757_at	PIK3IP1	1.7217	4.47E-07
242406_at	---	1.6994	7.93E-06
207571_x_at	C1orf38	1.6617	7.93E-06
212543_at	AIM1	1.6048	7.63E-05
244592_at	---	1.6022	9.01E-07
230599_at	---	1.5987	8.98E-08
231979_at	---	1.5849	7.55E-08
201700_at	CCND3	1.5810	7.55E-08
244357_at	---	1.5796	3.81E-05
214238_at	---	1.5760	1.91E-05
1568983_a_at	---	1.5696	7.55E-08
226218_at	IL7R	1.5694	2.96E-06
1555355_a_at	ETS1	1.5581	7.31E-05
212345_s_at	CREB3L2	1.5486	4.24E-07
225368_at	HIPK2	1.5454	3.98E-05
219028_at	HIPK2	1.5415	6.18E-03
227406_at	LOC100129387	1.5002	9.94E-06
203542_s_at	KLF9	1.4986	1.31E-05

Appendixes

Probe set	Gene symbol	LogRatio	FDR
230740_at	EHD3	1.4892	1.27E-04
219911_s_at	SLCO4A1	1.4879	1.29E-05
202239_at	PARP4	1.4783	3.78E-07
201009_s_at	TXNIP	1.4766	3.26E-04
201008_s_at	TXNIP	1.4721	2.03E-04
219888_at	SPAG4	1.4502	9.30E-06
203543_s_at	KLF9	1.4382	1.68E-05
222334_at	---	1.4097	3.21E-05
232722_at	RNASET2	1.3876	5.51E-05
242514_at	---	1.3767	3.33E-04
235274_at	---	1.3561	2.64E-06
219574_at	MARCH1	1.3557	8.67E-06
228891_at	SEMA4D	1.3356	3.75E-07
223085_at	RNF19A	1.3337	2.10E-07
203528_at	SEMA4D	1.3320	2.10E-07
204618_s_at	GABPB1	1.3204	2.64E-06
229822_at	---	1.3183	1.49E-04
242842_at	---	1.2627	2.56E-03
244026_at	---	1.2557	1.22E-03
216834_at	RGS1	1.2501	2.18E-05
204236_at	FLI1	1.2297	4.08E-05
225010_at	CCDC6	1.2284	3.65E-06
209681_at	SLC19A2	1.2232	2.57E-02
230381_at	C1orf186	1.2205	5.72E-05
228759_at	CREB3L2	1.1986	3.77E-05
224839_s_at	GPT2	1.1934	7.28E-05
229566_at	LOC645638	1.1926	6.41E-04
231109_at	---	1.1892	1.88E-05
213763_at	HIPK2	1.1873	9.27E-04
205510_s_at	FLJ10038	1.1746	2.95E-04
220483_s_at	RNF19A	1.1676	9.59E-05
239328_at	---	1.1631	9.30E-06
201161_s_at	CSDA	1.1618	4.62E-04
242551_at	---	1.1583	1.33E-04
224833_at	ETS1	1.1563	9.87E-06
219812_at	PVRIG	1.1416	7.99E-05
206173_x_at	GABPB1	1.1403	5.33E-05
227551_at	FAM108B1	1.1302	4.02E-07
239232_at	MSI2	1.1244	8.23E-04
226982_at	ELL2	1.1164	7.53E-04
223028_s_at	SNX9	1.1135	3.60E-05
37966_at	PARVB	1.1097	2.44E-04
240665_at	---	1.1077	3.16E-04
219753_at	STAG3	1.0989	6.34E-03
238393_at	---	1.0969	1.57E-03
242268_at	CELF2	1.0960	1.10E-03
1555372_at	BCL2L11	1.0909	4.37E-06
1555392_at	LOC100128868	1.0893	1.37E-03
201010_s_at	TXNIP	1.0873	2.37E-04
50221_at	TFEB	1.0702	3.60E-05
229670_at	---	1.0640	7.28E-05
231873_at	BMPR2	1.0536	9.90E-05
232583_at	---	1.0533	9.70E-05

Probe set	Gene symbol	LogRatio	FDR
228308_at	FKBP11	1.0521	1.26E-03
207734_at	LAX1	1.0483	3.66E-04
219117_s_at	FKBP11	1.0419	3.28E-03
230970_at	---	1.0409	2.16E-03
226099_at	ELL2	1.0403	9.28E-05
212400_at	FAM102A	1.0338	1.40E-03
208763_s_at	TSC22D3	1.0272	2.90E-04
230689_at	---	1.0225	8.68E-04
230536_at	PBX4	1.0155	2.68E-03
235385_at	MARCH1	1.0128	1.90E-04
236417_at	---	1.0118	2.53E-04
201367_s_at	ZFP36L2	1.0101	4.54E-02
217643_x_at	---	1.0100	7.93E-04
229383_at	MARCH1	1.0074	7.25E-05
237594_at	---	0.9981	6.43E-03
210786_s_at	FLI1	0.9940	1.82E-02
1557558_s_at	LOC100129196	0.9926	4.34E-05
242975_s_at	---	0.9911	1.01E-02
231775_at	TNFRSF10A	0.9862	1.31E-03
229501_s_at	USP8	0.9786	1.05E-05
214405_at	---	0.9769	5.90E-03
229274_at	GNAS	0.9760	1.89E-03
204716_at	CCDC6	0.9733	3.44E-04
237009_at	---	0.9690	2.08E-02
1560332_at	---	0.9682	3.28E-04
234151_at	---	0.9669	8.06E-04
1568943_at	INPP5D	0.9639	1.28E-02
211317_s_at	CFLAR	0.9560	4.37E-06
223027_at	SNX9	0.9560	5.67E-04
1570165_at	---	0.9481	4.08E-05
1558143_a_at	BCL2L11	0.9467	7.28E-05
213174_at	TTC9	0.9412	7.90E-04
219256_s_at	SH3TC1	0.9407	2.21E-03
224576_at	ERGIC1	0.9391	7.74E-03
202745_at	USP8	0.9364	9.92E-05
236341_at	CTLA4	0.9361	5.05E-03
209508_x_at	CFLAR	0.9344	9.87E-06
220612_at	---	0.9324	2.09E-02
231296_at	---	0.9298	1.17E-03
205882_x_at	ADD3	0.9295	8.37E-05
211862_x_at	CFLAR	0.9267	9.87E-06
201160_s_at	CSDA	0.9212	4.73E-06
208485_x_at	CFLAR	0.9205	9.87E-06
201369_s_at	ZFP36L2	0.9151	1.20E-02
215147_at	---	0.9147	1.32E-04
242827_x_at	---	0.9138	1.04E-03
202931_x_at	BIN1	0.9044	6.06E-04
210606_x_at	KLRD1	0.9040	4.14E-05
242946_at	---	0.8964	5.29E-04
244646_at	---	0.8933	7.82E-04
1554676_at	SRGN	0.8916	6.13E-03
239171_at	---	0.8901	1.45E-02
221773_at	ELK3	0.8858	1.89E-03

Appendixes

Probe set	Gene symbol	LogRatio	FDR
225606_at	BCL2L11	0.8851	5.25E-05
219118_at	FKBP11	0.8850	1.55E-02
241838_at	---	0.8843	4.14E-03
203315_at	NCK2	0.8787	3.60E-04
201752_s_at	ADD3	0.8754	1.29E-04
1561733_at	---	0.8749	9.19E-03
225282_at	SMAP2	0.8740	3.59E-04
203006_at	INPP5A	0.8701	3.84E-04
201938_at	CDK2AP1	0.8689	4.26E-04
225144_at	BMPR2	0.8678	2.22E-04
201753_s_at	ADD3	0.8643	1.51E-03
232864_s_at	AFF4	0.8609	3.11E-05
225562_at	RASA3	0.8580	1.16E-03
1569380_a_at	---	0.8570	5.47E-04
221193_s_at	ZCCHC10	0.8557	2.61E-04
239388_at	---	0.8534	6.92E-05
210564_x_at	CFLAR	0.8532	2.48E-05
1558747_at	SMCHD1	0.8528	9.13E-04
220285_at	FAM108B1	0.8487	1.92E-02
204484_at	PIK3C2B	0.8482	6.04E-04
201041_s_at	DUSP1	0.8450	1.34E-03
227312_at	SNTB2	0.8439	7.93E-04
201413_at	HSD17B4	0.8437	5.69E-04
232865_at	AFF4	0.8429	1.75E-04
1568997_at	---	0.8420	1.46E-03
232007_at	AGPAT5	0.8376	1.29E-02
221737_at	GNA12	0.8353	2.08E-03
240452_at	GSPT1	0.8350	3.59E-03
214439_x_at	BIN1	0.8349	2.16E-03
224681_at	GNA12	0.8346	2.22E-04
210201_x_at	BIN1	0.8219	1.45E-03
235668_at	PRDM1	0.8195	1.49E-04
225164_s_at	EIF2AK4	0.8189	7.05E-04
237018_at	---	0.8156	2.42E-02
203574_at	NFIL3	0.8144	9.00E-03
232623_at	LOC100128751	0.8133	9.37E-03
202158_s_at	CELF2	0.8117	3.87E-03
1566825_at	---	0.8116	3.93E-04
236458_at	---	0.8108	6.88E-04
212677_s_at	CEP68	0.8097	5.34E-03
240593_x_at	---	0.8092	2.73E-02
243509_at	---	0.8090	1.89E-02
214486_x_at	CFLAR	0.8083	5.84E-05
223803_s_at	ZCCHC10	0.8065	2.31E-04
1562600_at	---	0.8063	3.01E-03
232784_at	---	0.8051	2.85E-03
232542_at	COL9A2	0.8045	8.75E-05
226810_at	OGFRL1	0.8035	2.45E-02
244429_at	---	0.8020	9.84E-04
225763_at	RCSD1	0.8013	9.20E-03
233867_at	---	0.7924	2.21E-02
224577_at	ERGIC1	0.7904	3.82E-03
202912_at	ADM	0.7892	6.30E-03

Probe set	Gene symbol	LogRatio	FDR
235213_at	ITPKB	0.7860	9.25E-03
210563_x_at	CFLAR	0.7858	5.84E-05
213370_s_at	SFMBT1	0.7841	1.07E-04
235427_at	---	0.7836	1.41E-04
241837_at	---	0.7834	4.14E-03
237367_x_at	CFLAR	0.7814	3.45E-04
230590_at	---	0.7789	8.50E-03
203501_at	PGCP	0.7771	7.55E-03
219154_at	TMEM120B	0.7754	1.11E-02
1557557_at	LOC100129196	0.7746	4.44E-03
237426_at	SP100	0.7724	2.26E-03
222819_at	CTPS2	0.7659	7.28E-05
243395_at	---	0.7657	3.00E-03
241613_at	---	0.7654	1.34E-02
227146_at	QSOX2	0.7654	1.89E-05
211316_x_at	CFLAR	0.7647	8.80E-06
214157_at	GNAS	0.7626	2.24E-03
227999_at	PWWP2B	0.7608	3.13E-02
216253_s_at	PARVB	0.7593	1.39E-02
235421_at	MAP3K8	0.7556	2.07E-02
231794_at	CTLA4	0.7555	4.09E-03
235199_at	RNF125	0.7522	5.10E-03

APPENDIX 2

2.1. Probe sets down-regulated by dexamethasone in the high ZAP70 group

Probe set	Gene symbol	LogRatio	FDR
231093_at	FCRL3	-1.6818	8.94E-06
211138_s_at	KMO	-1.6749	2.58E-07
226694_at	AKAP2 /// PALM2-AKAP2	-1.6392	4.50E-06
226757_at	IFIT2	-1.6279	3.15E-05
227478_at	SETBP1	-1.5580	1.24E-05
222108_at	AMIGO2	-1.5426	5.99E-06
205681_at	BCL2A1	-1.5395	8.90E-06
205306_x_at	KMO	-1.5225	1.97E-07
226603_at	SAMD9L	-1.4927	1.03E-05
202759_s_at	AKAP2 /// PALM2-AKAP2	-1.4802	2.27E-05
204103_at	CCL4	-1.4703	8.65E-06
205483_s_at	ISG15	-1.4580	2.64E-04
219863_at	HERC5	-1.4481	3.77E-05
230036_at	SAMD9L	-1.4310	7.01E-06
202869_at	OAS1	-1.4115	5.12E-06
228599_at	MS4A1	-1.3955	3.71E-05
1569003_at	TMEM49	-1.3708	1.19E-04
216248_s_at	NR4A2	-1.3573	6.37E-06
209967_s_at	CREM	-1.3413	2.00E-06
202760_s_at	AKAP2 /// PALM2-AKAP2	-1.3194	2.10E-04
218986_s_at	DDX60	-1.3079	3.52E-05
230233_at	---	-1.2931	5.71E-06
207826_s_at	ID3	-1.2908	2.63E-06
224917_at	MIR21	-1.2863	3.72E-05
230511_at	CREM	-1.2804	3.52E-06
204622_x_at	NR4A2	-1.2656	1.20E-05
243271_at	---	-1.2629	8.01E-05
240498_at	---	-1.2555	2.08E-03
202086_at	MX1	-1.2553	3.81E-05
210279_at	GPR18	-1.2073	6.36E-07
200887_s_at	STAT1	-1.1927	5.42E-07
228617_at	XAF1	-1.1898	2.31E-05
202393_s_at	KLF10	-1.1896	1.42E-04
AFFX-HUMISGF3A/M97935_5_at	STAT1	-1.1804	3.57E-04
239544_at	---	-1.1770	1.76E-05
211192_s_at	CD84	-1.1680	6.90E-07
226142_at	GLIPR1	-1.1646	8.11E-06
203320_at	SH2B3	-1.1635	5.30E-05
200629_at	WARS	-1.1599	3.26E-05
228826_at	---	-1.1480	1.51E-05
209969_s_at	STAT1	-1.1421	5.30E-05
207630_s_at	CREM	-1.1414	3.23E-06
206126_at	CXCR5	-1.1343	5.77E-04
201560_at	CLIC4	-1.1320	2.85E-06
229968_at	---	-1.1318	1.14E-05
226748_at	LYSMD2	-1.1273	6.31E-07
AFFX-HUMISGF3A/M97935_MB_at	STAT1	-1.1256	1.23E-04

Probe set	Gene symbol	LogRatio	FDR
228531_at	SAMD9	-1.1195	1.64E-05
AFFX-HUMISGF3A/M97935_3_at	STAT1	-1.1134	6.12E-06
200628_s_at	WARS	-1.1046	3.01E-05
239294_at	---	-1.1009	2.28E-07
233085_s_at	OBFC2A	-1.0931	9.04E-07
214508_x_at	CREM	-1.0780	1.74E-07
202688_at	TNFSF10	-1.0538	1.31E-04
205861_at	SPIB	-1.0396	2.39E-03
219352_at	HERC6	-1.0362	2.19E-04
214677_x_at	CYAT1 /// IGLV1-44	-1.0331	1.58E-03
202446_s_at	PLSCR1	-1.0323	7.24E-04
229629_at	---	-1.0322	3.33E-06
201559_s_at	CLIC4	-1.0321	3.06E-03
223220_s_at	PARP9	-1.0305	5.92E-05
203932_at	HLA-DMB	-1.0300	7.40E-06
204908_s_at	BCL3	-1.0298	2.63E-04
213294_at	---	-1.0274	9.02E-05
204621_s_at	NR4A2	-1.0205	1.94E-04
204747_at	IFIT3	-1.0194	1.88E-02
224406_s_at	FCRL5	-1.0186	1.21E-06
204440_at	CD83	-1.0186	3.64E-05
235157_at	---	-1.0163	6.01E-04
222872_x_at	OBFC2A	-1.0126	3.64E-07
207339_s_at	LTB	-1.0110	5.49E-05
231418_at	---	-1.0091	4.49E-03
230391_at	CD84	-1.0055	6.13E-06
203140_at	BCL6	-1.0035	4.89E-05
205933_at	SETBP1	-0.9989	2.60E-04
203595_s_at	IFIT5	-0.9983	2.59E-04
205013_s_at	ADORA2A /// CYTSA	-0.9914	5.42E-07
203927_at	NFKBIE	-0.9882	4.26E-05
228298_at	FAM113B	-0.9827	7.07E-08
230805_at	---	-0.9820	3.04E-04
243798_at	---	-0.9808	5.81E-05
202499_s_at	SLC2A3	-0.9790	6.69E-06
201170_s_at	BHLHE40	-0.9773	1.39E-04
226136_at	GLIPR1	-0.9750	4.42E-05
208121_s_at	PTPRO	-0.9723	1.72E-04
242907_at	---	-0.9699	5.90E-04
238725_at	IRF1	-0.9698	4.07E-05
235276_at	EPSTI1	-0.9540	4.85E-05
205291_at	IL2RB	-0.9536	2.74E-06
204638_at	ACP5	-0.9512	1.47E-04
221087_s_at	APOL3	-0.9501	8.01E-05
224701_at	PARP14	-0.9493	8.14E-04
206133_at	XAF1	-0.9458	1.35E-04
1559776_at	---	-0.9440	6.64E-04
204238_s_at	C6orf108	-0.9438	5.42E-07
228152_s_at	DDX60L	-0.9426	3.30E-04
244447_at	---	-0.9396	5.20E-04
209138_x_at	IGL@	-0.9374	5.96E-04
202871_at	TRAF4	-0.9337	3.81E-03
219209_at	IFIH1	-0.9311	5.53E-05

Appendixes

Probe set	Gene symbol	LogRatio	FDR
225415_at	DTX3L	-0.9306	7.79E-05
219471_at	C13orf18	-0.9276	7.69E-06
226879_at	HVCN1	-0.9233	8.94E-06
213379_at	COQ2	-0.9177	3.64E-06
238429_at	TMEM71	-0.9145	2.25E-03
209959_at	NR4A3	-0.9142	5.66E-04
228758_at	BCL6	-0.9141	3.88E-04
215379_x_at	IGLV1-44	-0.9138	1.45E-03
219433_at	BCOR	-0.9126	1.52E-04
1566428_at	---	-0.9124	5.05E-05
215121_x_at	CYAT1 /// IGLV1-44	-0.9088	1.17E-03
202284_s_at	CDKN1A	-0.9041	2.45E-05
227844_at	FMNL3	-0.8987	2.87E-05
1557166_at	PDCD4	-0.8972	5.85E-04
212956_at	TBC1D9	-0.8964	1.70E-03
AFFX-HUMISGF3A/M97935_MA_at	STAT1	-0.8946	1.57E-03
238823_at	FMNL3	-0.8946	5.06E-05
205153_s_at	CD40	-0.8939	6.72E-04
214669_x_at	IGKC	-0.8935	1.43E-02
204994_at	MX2	-0.8927	5.12E-04
203879_at	PIK3CD	-0.8912	6.13E-06
219691_at	SAMD9	-0.8910	3.64E-04
235670_at	STX11	-0.8906	6.64E-05
210797_s_at	OASL	-0.8897	3.07E-03
212594_at	PDCD4	-0.8840	1.03E-05
204972_at	OAS2	-0.8784	3.30E-03
223222_at	SLC25A19	-0.8771	1.24E-05
226459_at	PIK3AP1	-0.8770	1.67E-06
214056_at	---	-0.8753	1.04E-02
211190_x_at	CD84	-0.8740	1.35E-04
218400_at	OAS3	-0.8731	6.52E-03
235170_at	ZNF92	-0.8724	4.53E-04
228828_at	---	-0.8707	1.04E-04
204221_x_at	GLIPR1	-0.8698	3.38E-04
201563_at	SORD	-0.8660	7.79E-05
206707_x_at	FAM65B	-0.8648	1.00E-05
220987_s_at	C11orf17 /// NUA2	-0.8599	3.60E-04
204032_at	BCAR3	-0.8597	4.18E-04
228837_at	TCF4	-0.8586	6.92E-05
209200_at	MEF2C	-0.8558	1.26E-05
228607_at	OAS2	-0.8538	5.95E-04
218611_at	IER5	-0.8527	6.69E-06
232375_at	---	-0.8509	8.12E-04
204222_s_at	GLIPR1	-0.8508	1.39E-04
217824_at	UBE2J1	-0.8480	5.81E-05
1557984_s_at	RPAP3	-0.8370	6.08E-06
226568_at	FAM102B	-0.8325	1.08E-04
227030_at	---	-0.8323	7.12E-05
227565_at	KLHL5	-0.8323	4.26E-05
225045_at	CCDC88A	-0.8310	2.16E-04
203144_s_at	KIAA0040	-0.8294	7.31E-04
220066_at	NOD2	-0.8282	3.54E-03
204998_s_at	ATF5	-0.8261	5.60E-04

Probe set	Gene symbol	LogRatio	FDR
220882_at	---	-0.8259	1.19E-06
216734_s_at	CXCR5	-0.8243	2.10E-03
203865_s_at	ADARB1	-0.8242	4.18E-04
225399_at	TSEN15	-0.8242	2.09E-06
214836_x_at	IGK@ /// IGKC	-0.8237	5.95E-03
241917_at	---	-0.8206	2.07E-04
208983_s_at	PECAM1	-0.8171	3.28E-04
205180_s_at	ADAM8	-0.8166	1.91E-04
228869_at	SNX20	-0.8162	1.21E-04
219258_at	TIPIN	-0.8160	6.42E-05
230052_s_at	NFKBID	-0.8146	2.88E-03
201631_s_at	IER3	-0.8144	3.58E-04
1561167_at	---	-0.8142	4.38E-04
212872_s_at	MED20	-0.8130	3.45E-06
216250_s_at	LPXN	-0.8106	1.44E-04
222808_at	ALG13	-0.8096	1.68E-05
1557966_x_at	MTERFD2	-0.8083	2.98E-06
1557965_at	MTERFD2	-0.8079	2.54E-06
1552291_at	PIGX	-0.8061	3.19E-03
209417_s_at	IFI35	-0.8032	8.14E-04
212048_s_at	YARS	-0.8021	1.02E-05
214085_x_at	GLIPR1	-0.7989	7.71E-05
208981_at	PECAM1	-0.7980	6.14E-05
219387_at	CCDC88A	-0.7966	2.64E-04
202748_at	GBP2	-0.7952	8.00E-05
242563_at	---	-0.7916	2.37E-03
1559263_s_at	PPIL4 /// ZC3H12D	-0.7892	4.59E-05
216041_x_at	GRN	-0.7876	1.88E-04
227211_at	PHF19	-0.7872	3.16E-05
219099_at	C12orf5	-0.7869	6.89E-06
44790_s_at	C13orf18	-0.7861	1.21E-05
215164_at	---	-0.7857	9.47E-05
206683_at	ZNF165	-0.7824	3.81E-05
227678_at	XRCC6BP1	-0.7819	1.36E-04
1554508_at	PIK3AP1	-0.7817	2.78E-03
209829_at	FAM65B	-0.7778	3.31E-04
230980_x_at	---	-0.7754	9.38E-05
229074_at	EHD4	-0.7720	3.35E-04
213638_at	PHACTR1	-0.7714	2.00E-04
227807_at	PARP9	-0.7704	9.43E-04
225285_at	BCAT1	-0.7698	2.55E-03
209199_s_at	MEF2C	-0.7692	3.81E-05
205205_at	RELB	-0.7679	9.52E-04
201761_at	MTHFD2	-0.7677	6.95E-05
213537_at	HLA-DPA1	-0.7666	1.52E-04
226045_at	FRS2	-0.7663	5.16E-05
221671_x_at	IGK@ /// IGKC	-0.7662	1.24E-02
200965_s_at	ABLIM1	-0.7650	1.13E-03
207375_s_at	IL15RA	-0.7648	3.76E-03
213224_s_at	NCRNA00081	-0.7626	3.28E-04
226264_at	SUSD1	-0.7624	1.88E-04
208103_s_at	ANP32E	-0.7609	2.31E-04
226841_at	MPEG1	-0.7606	1.00E-03

Probe set	Gene symbol	LogRatio	FDR
221651_x_at	IGK@ /// IGKC	-0.7595	1.13E-02
226117_at	TIFA	-0.7594	5.28E-06
230362_at	INPP5F	-0.7588	1.05E-04
215346_at	CD40	-0.7576	8.40E-04
205241_at	SCO2	-0.7576	4.34E-03
203046_s_at	TIMELESS	-0.7560	2.33E-05
208010_s_at	PTPN22	-0.7547	2.55E-03
206206_at	CD180	-0.7544	7.06E-05
242108_at	---	-0.7542	1.30E-03
212385_at	TCF4	-0.7538	5.77E-04
205019_s_at	VIPR1	-0.7518	2.36E-02
226784_at	TWISTNB	-0.7507	1.63E-03
1559391_s_at	---	-0.7504	1.12E-02

2.2. Probe sets down-regulated by dexamethasone in the low ZAP70 group

Probe set	Gene symbol	LogRatio	FDR
204103_at	CCL4	-2.1954	2.64E-06
202759_s_at	AKAP2 /// PALM2-AKAP2	-1.7259	6.46E-05
226694_at	AKAP2 /// PALM2-AKAP2	-1.6986	4.13E-05
205483_s_at	ISG15	-1.5873	1.22E-03
226603_at	SAMD9L	-1.5163	1.01E-04
202760_s_at	AKAP2 /// PALM2-AKAP2	-1.4944	7.21E-04
AFFX-HUMISGF3A/M97935_MB_at	STAT1	-1.4063	1.81E-04
211138_s_at	KMO	-1.3950	2.70E-05
224917_at	MIR21	-1.3921	1.90E-04
226757_at	IFIT2	-1.3785	1.27E-03
205681_at	BCL2A1	-1.3403	3.53E-04
205306_x_at	KMO	-1.3308	1.26E-05
230036_at	SAMD9L	-1.3059	1.90E-04
200628_s_at	WARS	-1.3030	7.41E-05
200629_at	WARS	-1.2985	1.27E-04
202086_at	MX1	-1.2960	2.99E-04
243271_at	---	-1.2921	6.55E-04
211192_s_at	CD84	-1.2843	4.73E-06
203751_x_at	JUND	-1.2737	2.52E-02
216248_s_at	NR4A2	-1.2637	1.39E-04
230805_at	---	-1.2627	3.64E-04
204908_s_at	BCL3	-1.2624	4.60E-04
205861_at	SPIB	-1.2445	5.24E-03
210279_at	GPR18	-1.2399	8.67E-06
1569003_at	TMEM49	-1.2388	2.39E-03
201559_s_at	CLIC4	-1.2384	6.59E-03
231093_at	FCRL3	-1.2332	1.41E-03
204747_at	IFIT3	-1.2217	3.75E-02
221239_s_at	FCRL2	-1.2185	1.00E-03
204415_at	IFI6	-1.2139	3.46E-02
202869_at	OAS1	-1.2081	2.44E-04
206126_at	CXCR5	-1.2062	3.00E-03
229629_at	---	-1.2032	1.05E-05
211190_x_at	CD84	-1.1983	8.90E-05

Probe set	Gene symbol	LogRatio	FDR
209969_s_at	STAT1	-1.1941	3.59E-04
AFFX-HUMISGF3A/M97935_5_at	STAT1	-1.1888	2.92E-03
AFFX-HUMISGF3A/M97935_MA_at	STAT1	-1.1727	1.73E-03
207826_s_at	ID3	-1.1653	8.75E-05
204622_x_at	NR4A2	-1.1645	2.81E-04
201631_s_at	IER3	-1.1634	1.75E-04
205241_at	SCO2	-1.1460	1.89E-03
208983_s_at	PECAM1	-1.1431	1.90E-04
200965_s_at	ABLIM1	-1.1393	4.37E-04
204858_s_at	TYMP	-1.1262	2.18E-03
200887_s_at	STAT1	-1.1197	1.55E-05
207113_s_at	TNF	-1.1191	1.28E-03
AFFX-HUMISGF3A/M97935_3_at	STAT1	-1.1107	7.35E-05
210972_x_at	TRA@ /// TRAC	-1.1086	3.73E-03
220987_s_at	C11orf17 /// NUA2	-1.1083	4.27E-04
223709_s_at	WNT10A	-1.1032	7.93E-04
204972_at	OAS2	-1.0953	5.54E-03
244352_at	CD84	-1.0939	1.39E-04
218400_at	OAS3	-1.0863	1.14E-02
226879_at	HVCN1	-1.0860	2.61E-05
223220_s_at	PARP9	-1.0806	3.93E-04
209671_x_at	TRA@ /// TRAC	-1.0707	1.24E-03
219471_at	C13orf18	-1.0677	2.76E-05
228599_at	MS4A1	-1.0669	3.08E-03
226568_at	FAM102B	-1.0651	1.30E-04
203835_at	LRR32	-1.0601	5.78E-04
204621_s_at	NR4A2	-1.0435	1.46E-03
228617_at	XAF1	-1.0344	7.93E-04
230391_at	CD84	-1.0337	5.83E-05
204238_s_at	C6orf108	-1.0303	3.78E-06
228055_at	NAPSB	-1.0255	1.44E-02
209417_s_at	IFI35	-1.0232	1.10E-03
228826_at	---	-1.0222	4.58E-04
216734_s_at	CXCR5	-1.0180	3.63E-03
201560_at	CLIC4	-1.0156	9.70E-05
202730_s_at	PDCD4	-1.0151	3.51E-02
219863_at	HERC5	-1.0086	6.02E-03
206133_at	XAF1	-1.0086	7.69E-04
228837_at	TCF4	-1.0070	1.74E-04
224701_at	PARP14	-1.0052	4.25E-03
232375_at	---	-1.0039	1.97E-03
205291_at	IL2RB	-1.0010	2.44E-05
202531_at	IRF1	-0.9989	3.47E-03
214508_x_at	CREM	-0.9912	7.93E-06
204440_at	CD83	-0.9900	4.62E-04
208981_at	PECAM1	-0.9876	9.59E-05
223222_at	SLC25A19	-0.9875	5.23E-05
207630_s_at	CREM	-0.9852	1.50E-04
205599_at	TRAF1	-0.9786	4.17E-04
213294_at	---	-0.9773	1.29E-03
202688_at	TNFSF10	-0.9720	2.26E-03
204994_at	MX2	-0.9708	2.27E-03
216250_s_at	LPXN	-0.9676	3.22E-04

Appendixes

Probe set	Gene symbol	LogRatio	FDR
205013_s_at	ADORA2A /// CYTSA	-0.9662	1.05E-05
228828_at	---	-0.9655	4.30E-04
235372_at	FCRLA	-0.9642	7.93E-06
216236_s_at	SLC2A14 /// SLC2A3	-0.9601	9.55E-03
218986_s_at	DDX60	-0.9600	3.93E-03
230511_at	CREM	-0.9592	5.69E-04
209967_s_at	CREM	-0.9492	5.53E-04
202446_s_at	PLSCR1	-0.9469	1.00E-02
205988_at	CD84	-0.9436	1.14E-04
239740_at	ETV6	-0.9432	1.70E-02
204638_at	ACP5	-0.9431	1.45E-03
202748_at	GBP2	-0.9399	1.90E-04
205205_at	RELB	-0.9385	1.75E-03
239294_at	---	-0.9379	1.90E-05
201341_at	ENC1	-0.9296	1.33E-04
211269_s_at	IL2RA	-0.9287	3.77E-03
203932_at	HLA-DMB	-0.9266	2.30E-04
208982_at	PECAM1	-0.9248	7.81E-05
212956_at	TBC1D9	-0.9245	1.00E-02
221671_x_at	IGK@ /// IGKC	-0.9240	2.48E-02
221651_x_at	IGK@ /// IGKC	-0.9240	2.19E-02
228298_at	FAM113B	-0.9231	2.64E-06
227030_at	---	-0.9221	2.96E-04
209670_at	TRAC	-0.9207	1.35E-04
203140_at	BCL6	-0.9186	1.02E-03
235276_at	EPSTI1	-0.9182	6.72E-04
242907_at	---	-0.9178	6.84E-03
222108_at	AMIGO2	-0.9157	4.66E-03
1554508_at	PIK3AP1	-0.9146	7.09E-03
212671_s_at	HLA-DQA1 /// HLA-DQA2	-0.9136	3.01E-02
203143_s_at	KIAA0040	-0.9129	2.97E-03
210797_s_at	OASL	-0.9119	1.72E-02
215223_s_at	SOD2	-0.9098	1.09E-03
201490_s_at	PPIF	-0.9087	2.01E-03
219352_at	HERC6	-0.9041	5.17E-03
1554834_a_at	RASSF5	-0.9008	2.03E-02
228056_s_at	NAPSB	-0.8995	9.92E-03
202393_s_at	KLF10	-0.8975	9.66E-03
224406_s_at	FCRL5	-0.8955	5.84E-05
215990_s_at	BCL6	-0.8955	3.58E-03
204269_at	PIM2	-0.8940	1.39E-02
206759_at	FCER2	-0.8933	1.83E-02
205153_s_at	CD40	-0.8932	5.35E-03
207968_s_at	MEF2C	-0.8889	8.38E-03
224193_s_at	FCRL2	-0.8829	3.26E-04
201601_x_at	IFITM1	-0.8823	1.50E-02
221044_s_at	TRIM34 /// TRIM6-TRIM34	-0.8809	2.12E-02
208010_s_at	PTPN22	-0.8799	6.60E-03
219691_at	SAMD9	-0.8794	3.44E-03
202087_s_at	CTSL1	-0.8767	1.95E-03
227609_at	EPSTI1	-0.8763	2.22E-04
203072_at	MYO1E	-0.8757	9.59E-05
211430_s_at	IGH@ /// IGHG1	-0.8757	2.01E-02

Probe set	Gene symbol	LogRatio	FDR
228531_at	SAMD9	-0.8742	1.41E-03
202499_s_at	SLC2A3	-0.8739	2.22E-04
206760_s_at	FCER2	-0.8730	1.22E-02
214677_x_at	CYAT1 /// IGLV1-44	-0.8718	2.85E-02
203595_s_at	IFIT5	-0.8716	5.94E-03
209636_at	NFKB2	-0.8716	2.44E-02
1559776_at	---	-0.8702	9.00E-03
203471_s_at	PLEK	-0.8698	2.58E-04
205180_s_at	ADAM8	-0.8666	1.10E-03
225447_at	GPD2	-0.8643	9.50E-04
235157_at	---	-0.8598	1.40E-02
235670_at	STX11	-0.8581	8.79E-04
1552497_a_at	SLAMF6	-0.8559	4.79E-03
236280_at	---	-0.8558	3.60E-05
206341_at	IL2RA	-0.8532	1.70E-02
226748_at	LYSMD2	-0.8519	1.27E-04
211725_s_at	BID	-0.8492	9.29E-05
203320_at	SH2B3	-0.8490	5.61E-03
239979_at	---	-0.8474	6.95E-04
216841_s_at	SOD2	-0.8461	3.98E-04
206150_at	CD27	-0.8437	7.93E-04
213620_s_at	ICAM2	-0.8435	9.17E-04
229968_at	---	-0.8386	1.53E-03
238725_at	IRF1	-0.8382	1.34E-03
1563674_at	FCRL2	-0.8369	1.11E-03
224795_x_at	IGK@ /// IGKC	-0.8366	4.40E-02
227807_at	PARP9	-0.8332	4.20E-03
200824_at	GSTP1	-0.8322	4.12E-03
208121_s_at	PTPRO	-0.8308	4.87E-03
221087_s_at	APOL3	-0.8308	2.21E-03
228518_at	IGH@ /// IGHG1 /// IGHM	-0.8303	4.67E-02
227478_at	SETBP1	-0.8282	1.54E-02
214836_x_at	IGK@ /// IGKC	-0.8244	3.37E-02
1561167_at	---	-0.8237	3.39E-03
213261_at	TRANK1	-0.8221	7.72E-03
44790_s_at	C13orf18	-0.8220	9.28E-05
209138_x_at	IGL@	-0.8215	1.12E-02
212960_at	TBC1D9	-0.8208	2.92E-02
228758_at	BCL6	-0.8173	7.10E-03
223903_at	TLR9	-0.8152	1.41E-03
221449_s_at	ITFG1	-0.8149	1.98E-03
226117_at	TIFA	-0.8130	3.60E-05
209200_at	MEF2C	-0.8111	2.31E-04
203523_at	LSP1	-0.8093	2.00E-02
238823_at	FMNL3	-0.8032	1.20E-03
201563_at	SORD	-0.8007	1.39E-03
224990_at	C4orf34	-0.7971	3.15E-03
201251_at	PKM2	-0.7969	1.78E-02
201105_at	LGALS1	-0.7930	2.26E-03
203927_at	NFKBIE	-0.7918	2.41E-03
203045_at	NINJ1	-0.7913	1.37E-03
212641_at	HIVEP2	-0.7884	1.09E-03
204683_at	ICAM2	-0.7868	1.98E-03

Appendixes

Probe set	Gene symbol	LogRatio	FDR
211902_x_at	TRA@	-0.7853	7.51E-03
201761_at	MTHFD2	-0.7844	5.67E-04
214995_s_at	APOBEC3F /// APOBEC3G	-0.7840	2.95E-04
231769_at	FBXO6	-0.7810	6.79E-03
221078_s_at	CCDC88A	-0.7807	1.58E-02
226142_at	GLIPR1	-0.7806	2.56E-03
208680_at	PRDX1	-0.7771	2.19E-02
226408_at	TEAD2	-0.7760	2.94E-02
207419_s_at	RAC2	-0.7733	4.36E-03
211991_s_at	HLA-DPA1	-0.7723	1.04E-02
201649_at	UBE2L6	-0.7698	7.72E-03
224574_at	C17orf49	-0.7697	1.55E-03
220054_at	IL23A	-0.7684	1.80E-04
204882_at	ARHGAP25	-0.7682	4.87E-03
211189_x_at	CD84	-0.7649	7.52E-04
228152_s_at	DDX60L	-0.7629	1.17E-02
243798_at	---	-0.7625	3.87E-03
219690_at	TMEM149	-0.7613	1.56E-03
1554667_s_at	METTL8	-0.7602	5.58E-04
220066_at	NOD2	-0.7593	3.44E-02
38149_at	ARHGAP25	-0.7578	2.54E-03
222088_s_at	SLC2A14 /// SLC2A3	-0.7571	6.76E-03
225974_at	TMEM64	-0.7562	5.06E-03
201000_at	AARS	-0.7545	1.83E-03
210550_s_at	RASGRF1	-0.7540	2.36E-02
206060_s_at	PTPN22	-0.7539	1.04E-03
212998_x_at	HLA-DQB1 /// LOC100294318	-0.7522	7.83E-03
218966_at	MYO5C	-0.7513	2.98E-03
207535_s_at	NFKB2	-0.7509	1.92E-02

APPENDIX 3

3.1. Probe sets with higher expression in the untreated cells of the low ZAP70 group

Probe set	Gene symbol	LogRatio	FDR
227082_at	---	3.1500	5.02E-05
212560_at	SORL1	3.0682	2.16E-03
213158_at	---	3.0415	4.41E-06
213156_at	---	2.9626	4.41E-06
227121_at	---	2.9399	5.44E-06
240216_at	---	2.8393	4.41E-06
205383_s_at	ZBTB20	2.7978	4.41E-06
235150_at	---	2.7861	8.31E-03
203509_at	SORL1	2.7860	3.52E-03
235308_at	ZBTB20	2.7745	4.41E-06
215967_s_at	LY9	2.7478	6.64E-03
226252_at	---	2.7173	1.78E-05
210370_s_at	LY9	2.6964	4.26E-03
231124_x_at	LY9	2.5399	8.95E-03
235683_at	SESN3	2.5228	7.43E-04
242134_at	---	2.4825	5.41E-04
209959_at	NR4A3	2.4748	5.44E-03
226250_at	---	2.4231	1.33E-04
214508_x_at	CREM	2.1956	1.29E-03
243546_at	---	2.1808	6.10E-03
227613_at	ZNF331	2.1554	2.39E-03
209967_s_at	CREM	2.1382	6.22E-03
235213_at	ITPKB	2.1331	1.24E-02
203835_at	LRR32	2.1037	2.44E-02
204334_at	KLF7	2.1000	3.38E-03
230511_at	CREM	2.0835	6.22E-03
207630_s_at	CREM	2.0617	2.16E-03
204621_s_at	NR4A2	2.0222	3.15E-03
219228_at	ZNF331	2.0104	8.13E-04
229566_at	LOC645638	1.9884	9.76E-03
216248_s_at	NR4A2	1.9072	3.52E-03
212609_s_at	AKT3	1.8483	2.89E-02
37145_at	GNLY	1.8150	2.13E-02
235739_at	---	1.7767	1.14E-03
226039_at	MGAT4A	1.7403	1.24E-02
204622_x_at	NR4A2	1.7305	5.85E-03
214657_s_at	NEAT1	1.7250	7.11E-03
227062_at	---	1.7068	1.76E-02
230536_at	PBX4	1.6184	1.87E-02
205495_s_at	GNLY	1.6130	3.23E-02
213915_at	NKG7	1.5472	3.56E-02
211458_s_at	GABARAPL1 /// GABARAPL3	1.5195	3.39E-03
238320_at	---	1.4963	3.89E-02
204761_at	USP6NL	1.4799	8.31E-03
214470_at	KLRB1	1.4744	1.94E-02
1554544_a_at	MBP	1.4742	8.19E-03
228528_at	LOC100286909	1.4304	3.94E-03

Appendixes

Probe set	Gene symbol	LogRatio	FDR
243918_at	---	1.4296	8.31E-03
241015_at	---	1.3993	4.51E-02
201422_at	IFI30	1.3932	3.31E-02
210136_at	MBP	1.3848	1.37E-02
209626_s_at	OSBPL3	1.3494	2.36E-02
212099_at	RHOB	1.3438	3.59E-02
212658_at	LHFPL2	1.3293	7.86E-03
210606_x_at	KLRD1	1.3219	2.11E-02
225252_at	SRXN1	1.2951	2.47E-02
212796_s_at	TBC1D2B	1.2951	9.04E-04
1560397_s_at	KLHL6	1.2914	1.24E-02
201041_s_at	DUSP1	1.2844	7.43E-04
210031_at	CD247	1.2803	3.57E-02
220132_s_at	CLEC2D	1.2798	3.99E-02
201466_s_at	JUN	1.2769	1.17E-02
208869_s_at	GABARAPL1	1.2729	9.65E-03
233899_x_at	ZBTB10	1.2431	2.97E-02
233500_x_at	CLEC2D	1.2013	2.99E-02
214446_at	ELL2	1.1940	1.75E-02
225553_at	---	1.1899	4.55E-03
207072_at	IL18RAP	1.1743	3.04E-02
224832_at	DUSP16	1.1723	2.59E-02
203723_at	ITPKB	1.1705	8.31E-03
212636_at	QKI	1.1668	3.62E-02
57540_at	RBKS	1.1583	1.04E-02
224566_at	NEAT1	1.1580	6.34E-03
201464_x_at	JUN	1.1578	4.04E-02
219312_s_at	ZBTB10	1.1538	3.23E-02
241985_at	JMY	1.1396	1.38E-02
206966_s_at	KLF12	1.1313	1.58E-02
225123_at	---	1.1277	1.01E-02
201531_at	ZFP36	1.1170	2.03E-03
205590_at	RASGRP1	1.1117	3.88E-02
206917_at	GNA13	1.1115	2.16E-03
1554690_a_at	TACC1	1.1047	3.67E-02
215707_s_at	PRNP	1.0901	2.47E-02
202364_at	MXI1	1.0897	7.11E-04
1555827_at	CCNL1	1.0804	2.42E-02
202887_s_at	DDIT4	1.0797	2.95E-02
214786_at	MAP3K1	1.0770	2.87E-02
212509_s_at	MXRA7	1.0738	3.34E-03
225651_at	UBE2E2	1.0685	3.06E-02
226440_at	DUSP22	1.0676	3.98E-02
216976_s_at	RYK	1.0589	4.80E-02
205434_s_at	AAK1	1.0546	2.47E-02
204222_s_at	GLIPR1	1.0539	8.19E-03
224978_s_at	USP36	1.0316	1.65E-02
228167_at	KLHL6	1.0280	1.01E-02
239912_at	---	1.0174	3.36E-03
201465_s_at	JUN	1.0165	3.21E-02
207001_x_at	TSC22D3	1.0141	1.70E-02
226989_at	RGMB	1.0105	7.89E-03
201751_at	JOSD1	1.0104	7.43E-04

Probe set	Gene symbol	LogRatio	FDR
202255_s_at	SIPA1L1	1.0075	4.99E-03
226142_at	GLIPR1	0.9891	3.48E-02
224601_at	---	0.9886	3.11E-02
219222_at	RBKS	0.9844	4.03E-03
227539_at	GNA13	0.9663	6.50E-03
212676_at	NF1	0.9561	8.19E-03
244804_at	SQSTM1	0.9352	4.05E-02
215013_s_at	USP34	0.9343	2.47E-02
1558407_at	PLEKHG2	0.9302	9.16E-03
201653_at	CNIH	0.9287	9.26E-04
209331_s_at	MAX	0.9285	1.71E-02
202899_s_at	SFRS3	0.9262	9.75E-03
208868_s_at	GABARAPL1	0.9086	8.95E-03
229312_s_at	GKAP1	0.9040	4.05E-02
202466_at	PAPD7	0.9016	2.95E-02
221986_s_at	KLHL24	0.9004	4.94E-02
226352_at	JMY	0.9002	3.15E-03
239845_at	---	0.8796	3.57E-03
209383_at	DDIT3	0.8787	7.86E-03
224565_at	NEAT1	0.8780	8.19E-03
207920_x_at	ZFX	0.8751	2.18E-02
227093_at	USP36	0.8728	5.40E-03
237746_at	SFRS11	0.8689	1.86E-02
214429_at	MTMR6	0.8677	1.29E-02
226136_at	GLIPR1	0.8660	3.65E-02
202254_at	SIPA1L1	0.8652	3.91E-02
235536_at	SNORD89	0.8638	1.70E-03
1558111_at	MBNL1	0.8618	1.94E-02
221985_at	KLHL24	0.8532	1.59E-02
219507_at	RSRC1	0.8478	3.43E-02
1552611_a_at	JAK1	0.8450	2.42E-02
208763_s_at	TSC22D3	0.8396	1.06E-03
207351_s_at	SH2D2A	0.8360	4.04E-02
223376_s_at	BRI3	0.8337	1.75E-02
201471_s_at	SQSTM1	0.8258	9.98E-03
205411_at	STK4	0.8252	3.32E-02
1558517_s_at	LRR8C	0.8239	4.04E-02
206809_s_at	HNRNPA3 /// HNRNPA3P1	0.8230	4.01E-02
226679_at	SLC26A11	0.8167	3.12E-02
211085_s_at	STK4	0.8132	1.89E-02
211932_at	HNRNPA3	0.8086	1.68E-02
218329_at	PRDM4	0.8064	6.32E-03
207113_s_at	TNF	0.8048	1.86E-02
211610_at	KLF6	0.8029	1.87E-02
203925_at	GCLM	0.8001	1.87E-02
208961_s_at	KLF6	0.7963	4.57E-02
1556385_at	---	0.7945	2.50E-02
49485_at	PRDM4	0.7795	8.19E-03
219497_s_at	BCL11A	0.7747	4.45E-02
208622_s_at	EZR	0.7739	4.36E-02
207485_x_at	BTN3A1	0.7719	3.00E-02
1552610_a_at	JAK1	0.7694	7.88E-03
213198_at	ACVR1B	0.7620	8.65E-03

Probe set	Gene symbol	LogRatio	FDR
1557502_at	PCCB	0.7609	3.72E-03
201101_s_at	BCLAF1	0.7583	4.76E-02
210115_at	RPL39L	0.7583	5.91E-03
1555465_at	MCOLN2	0.7576	4.37E-02

3.2. Probe sets with higher expression in the untreated cells of the high ZAP70 group

Probe set	Gene symbol	LogRatio	FDR
233483_at	TBC1D27	3.1343	1.33E-04
205204_at	NMB	2.7659	4.04E-03
225285_at	BCAT1	2.5234	3.72E-03
226517_at	BCAT1	2.4014	5.91E-03
228532_at	C1orf162	2.2651	3.40E-04
215925_s_at	CD72	2.2206	3.57E-03
242572_at	---	2.1691	4.41E-02
219256_s_at	SH3TC1	1.8763	3.23E-02
212504_at	DIP2C	1.8061	9.65E-03
238559_at	---	1.7712	8.13E-04
207761_s_at	METTL7A	1.7661	1.95E-03
213839_at	CLMN	1.7425	8.13E-04
209732_at	CLEC2B	1.7348	4.81E-02
219518_s_at	ELL3 /// SERINC4	1.6656	7.43E-04
201850_at	CAPG	1.6430	2.18E-04
203186_s_at	S100A4	1.6340	1.40E-02
202283_at	SERPINF1	1.5758	4.76E-02
226134_s_at	---	1.5707	9.33E-03
203711_s_at	HIBCH	1.5491	5.40E-03
230362_at	INPP5F	1.5461	8.22E-03
219517_at	ELL3 /// SERINC4	1.5344	4.75E-04
218100_s_at	IFT57	1.5225	1.39E-04
1555613_a_at	ZAP70	1.5139	8.23E-03
214032_at	ZAP70	1.5117	3.91E-03
205267_at	POU2AF1	1.5092	3.72E-03
216620_s_at	ARHGEF10	1.5091	2.18E-02
225240_s_at	MSI2	1.4918	8.78E-03
204135_at	FILIP1L	1.4898	3.99E-02
244050_at	PTPLAD2	1.4821	2.96E-03
230983_at	FAM129C	1.4747	1.55E-02
201037_at	PFKP	1.4718	5.07E-04
205081_at	CRIP1	1.4458	1.73E-02
208614_s_at	FLNB	1.4263	4.99E-03
210785_s_at	C1orf38	1.4130	9.26E-03
213374_x_at	HIBCH	1.4102	9.04E-03
210609_s_at	TP53I3	1.4095	1.87E-03
1555379_at	FAM159A	1.4011	3.59E-02
210448_s_at	P2RX5	1.3972	7.43E-04
205677_s_at	DLEU1	1.3889	2.18E-02
230363_s_at	INPP5F	1.3817	3.15E-02
237400_at	ATP5S	1.3806	1.37E-02
220016_at	AHNAK	1.3737	4.99E-03

Probe set	Gene symbol	LogRatio	FDR
214366_s_at	ALOX5	1.3699	3.52E-03
226358_at	APH1B	1.3583	7.43E-04
207571_x_at	C1orf38	1.3582	8.23E-03
214238_at	---	1.3523	8.90E-05
229437_at	MIR155HG	1.3484	9.75E-03
235372_at	FCRLA	1.3381	4.99E-03
224990_at	C4orf34	1.3337	1.24E-02
224789_at	DCAF12	1.3267	7.06E-04
207339_s_at	LTB	1.3264	3.13E-02
232773_at	---	1.3248	8.31E-03
228056_s_at	NAPSB	1.3203	1.44E-02
236316_at	FAM3C	1.3199	3.64E-02
227052_at	---	1.2999	6.55E-03
225624_at	SNX29	1.2955	5.29E-04
1553906_s_at	FGD2	1.2891	2.45E-03
236539_at	PTPN22	1.2796	1.86E-02
224989_at	---	1.2633	4.33E-02
242722_at	LMO7	1.2594	7.44E-03
229130_at	---	1.2594	3.94E-03
225757_s_at	CLMN	1.2562	1.29E-03
201760_s_at	WSB2	1.2451	8.22E-03
230131_x_at	ARSD	1.2428	1.11E-02
225637_at	DEF8	1.2358	7.73E-03
1558185_at	CLLU1	1.2329	2.97E-02
205801_s_at	RASGRP3	1.2285	1.87E-03
223553_s_at	DOK3	1.1982	1.54E-03
219173_at	MYO15B	1.1926	3.80E-02
204446_s_at	ALOX5	1.1917	3.52E-03
225164_s_at	EIF2AK4	1.1886	1.40E-02
243154_at	---	1.1829	1.42E-03
207641_at	TNFRSF13B	1.1800	7.73E-03
225382_at	ZNF275	1.1726	7.73E-03
205462_s_at	HPCAL1	1.1720	2.47E-02
206150_at	CD27	1.1634	1.51E-02
204257_at	FADS3	1.1634	3.41E-02
235459_at	---	1.1494	8.22E-03
224735_at	CYBASC3	1.1483	4.22E-04
216041_x_at	GRN	1.1464	1.52E-02
59375_at	MYO15B	1.1455	2.90E-02
227182_at	SUSD3	1.1415	3.52E-03
226419_s_at	FLJ44342	1.1349	2.47E-02
202942_at	ETFB	1.1349	4.50E-04
205547_s_at	TAGLN	1.1324	6.50E-03
229958_at	CLN8	1.1145	3.34E-02
1552807_a_at	SIGLEC10 /// SIGLEC12	1.1111	2.12E-02
212611_at	DTX4	1.0983	1.79E-02
209703_x_at	METTL7A	1.0929	2.23E-03
1568658_at	C2orf74	1.0918	1.39E-02
200678_x_at	GRN	1.0912	1.93E-02
205353_s_at	PEBP1	1.0865	8.19E-03
236198_at	---	1.0856	8.22E-03
35974_at	LRMP	1.0818	2.30E-02
1558345_a_at	LOC439911	1.0743	1.17E-02

Appendixes

Probe set	Gene symbol	LogRatio	FDR
212552_at	HPCAL1	1.0659	1.17E-02
225311_at	IVD	1.0519	8.56E-03
208613_s_at	FLNB	1.0480	1.69E-02
211284_s_at	GRN	1.0408	1.78E-02
210825_s_at	PEBP1	1.0331	5.85E-03
204445_s_at	ALOX5	1.0295	8.49E-03
221036_s_at	APH1B	1.0251	4.70E-04
203921_at	CHST2	1.0217	6.11E-03
1565752_at	FGD2	1.0182	4.99E-03
205011_at	VWA5A	1.0169	2.47E-02
235512_at	CDKL1	1.0145	8.19E-03
204396_s_at	GRK5	1.0114	1.40E-02
210944_s_at	CAPN3	1.0114	3.52E-02
205718_at	ITGB7	1.0114	1.75E-02
213888_s_at	TRAF3IP3	1.0103	4.47E-02
205484_at	SIT1	1.0054	4.03E-03
206060_s_at	PTPN22	1.0021	2.79E-02
1565754_x_at	FGD2	1.0016	6.34E-03
229803_s_at	---	0.9999	3.04E-02
227943_at	---	0.9990	2.53E-02
1570505_at	ABCB4	0.9941	3.08E-02
208998_at	UCP2	0.9940	4.99E-03
211986_at	AHNAK	0.9933	1.86E-02
205901_at	PNOC	0.9917	7.54E-04
205297_s_at	CD79B	0.9885	3.40E-02
212913_at	C6orf26	0.9879	2.13E-02
204401_at	KCNN4	0.9879	1.04E-02
223686_at	TPK1	0.9852	1.16E-02
221666_s_at	PYCARD	0.9839	3.90E-02
226333_at	IL6R	0.9694	3.22E-02
203607_at	INPP5F	0.9689	4.09E-02
222519_s_at	IFT57	0.9650	9.98E-03
210889_s_at	FCGR2B	0.9606	4.27E-02
226608_at	C16orf87	0.9595	8.55E-03
224840_at	FKBP5	0.9580	1.29E-02
1552634_a_at	ZNF101	0.9535	2.74E-02
229114_at	GAB1	0.9432	4.78E-02
216958_s_at	IVD	0.9401	1.24E-02
1553369_at	FAM129C	0.9332	2.60E-02
225383_at	ZNF275	0.9326	2.47E-02
203358_s_at	EZH2	0.9320	2.21E-02
204674_at	LRMP	0.9319	3.98E-02
227111_at	ZBTB34	0.9291	6.47E-03
228994_at	CCDC24	0.9280	3.52E-03
213734_at	WSB2	0.9277	4.58E-02
225387_at	TSPAN5	0.9254	4.52E-02
201029_s_at	CD99	0.9227	6.68E-03
218597_s_at	CISD1	0.9214	3.36E-03
227607_at	STAMBPL1	0.9211	4.99E-03
235580_at	ZNF141	0.9202	3.15E-02
210184_at	ITGAX	0.9187	2.23E-02
226673_at	SH2D3C	0.9041	9.65E-03
204929_s_at	VAMP5	0.9028	3.49E-02

Probe set	Gene symbol	LogRatio	FDR
223299_at	SEC11C	0.9024	8.62E-04
221782_at	DNAJC10	0.9010	1.86E-02
227198_at	AFF3	0.9003	3.72E-02
229214_at	---	0.8946	3.21E-02
221218_s_at	TPK1	0.8938	8.31E-03
207843_x_at	CYB5A	0.8843	3.61E-02
74694_s_at	RABEP2	0.8817	2.18E-02
242260_at	MATR3	0.8811	6.83E-03
229937_x_at	LILRB1	0.8710	3.08E-02
211941_s_at	PEBP1	0.8692	2.64E-02
1555724_s_at	TAGLN	0.8653	8.19E-03
226795_at	LRCH1	0.8622	1.11E-02
202329_at	CSK	0.8595	8.87E-04
213280_at	RAP1GAP2	0.8581	4.05E-02
227606_s_at	STAMBPL1	0.8563	2.16E-02
212605_s_at	---	0.8539	1.01E-02
201825_s_at	SCCPDH	0.8492	2.79E-02
221058_s_at	CKLF	0.8482	4.52E-02
238920_at	---	0.8415	4.81E-02
230708_at	PRICKLE1	0.8364	2.51E-02
229597_s_at	WDFY4	0.8363	3.34E-02
225723_at	C6orf129	0.8343	2.47E-02
222762_x_at	LIMD1	0.8298	4.04E-02
227840_at	C2orf76	0.8297	4.78E-02
202207_at	ARL4C	0.8259	2.66E-02
211890_x_at	CAPN3	0.8254	1.01E-02
203421_at	TP53I11	0.8125	7.88E-03
228869_at	SNX20	0.8123	9.65E-03
208690_s_at	PDLIM1	0.8066	3.43E-03
222942_s_at	TIAM2	0.8022	8.31E-03
213600_at	SIPA1L3	0.8003	8.19E-03
229750_at	POU2F2	0.7897	4.80E-02
201813_s_at	TBC1D5	0.7885	3.98E-02
201487_at	CTSC	0.7880	1.05E-02
204199_at	RALGPS1	0.7854	3.52E-03
205049_s_at	CD79A	0.7848	5.06E-03
242556_at	---	0.7847	2.30E-02
213857_s_at	CD47	0.7774	1.81E-04
218557_at	NIT2	0.7764	3.60E-03
206272_at	RAB4A /// SPHAR	0.7733	1.40E-02
203932_at	HLA-DMB	0.7731	5.82E-03
229681_at	---	0.7731	6.75E-03
215947_s_at	FAM136A	0.7702	1.91E-02
1563641_a_at	SNX20	0.7659	2.47E-02
217824_at	UBE2J1	0.7651	1.28E-02
223514_at	CARD11	0.7610	1.29E-02
213188_s_at	MINA	0.7608	4.52E-02
228956_at	UGT8	0.7577	1.15E-02
217478_s_at	HLA-DMA	0.7563	4.87E-03
225332_at	LOC729082	0.7507	4.17E-02

APPENDIX 4

4.1. Probe sets with higher expression in the treated cells of the low ZAP70 group

Probe set	Gene symbol	LogRatio	FDR
227082_at	---	3.3666	1.21E-05
213156_at	---	3.1192	3.75E-06
213158_at	---	3.0209	3.75E-06
240216_at	---	2.8672	4.57E-06
209959_at	NR4A3	2.8387	1.02E-03
226252_at	---	2.7440	1.16E-05
212560_at	SORL1	2.7142	3.44E-03
215967_s_at	LY9	2.6743	5.26E-03
205383_s_at	ZBTB20	2.6490	9.39E-06
235308_at	ZBTB20	2.6056	9.39E-06
227121_at	---	2.5575	2.99E-05
229566_at	LOC645638	2.5304	1.02E-03
209967_s_at	CREM	2.5302	9.43E-04
226250_at	---	2.4515	6.33E-05
210370_s_at	LY9	2.4483	5.50E-03
230511_at	CREM	2.4047	1.22E-03
203509_at	SORL1	2.4019	6.13E-03
231124_x_at	LY9	2.3588	1.01E-02
214508_x_at	CREM	2.2824	5.03E-04
207630_s_at	CREM	2.2179	6.03E-04
201466_s_at	JUN	2.1147	9.01E-05
204334_at	KLF7	2.0830	2.07E-03
216248_s_at	NR4A2	2.0008	1.30E-03
204621_s_at	NR4A2	1.9991	1.93E-03
235683_at	SESN3	1.9448	3.91E-03
242134_at	---	1.9201	2.98E-03
212609_s_at	AKT3	1.8824	1.76E-02
204761_at	USP6NL	1.8709	8.40E-04
219228_at	ZNF331	1.8687	1.02E-03
202912_at	ADM	1.8640	4.08E-02
204622_x_at	NR4A2	1.8317	2.42E-03
227613_at	ZNF331	1.7724	6.13E-03
235739_at	---	1.7717	7.09E-04
1559421_at	---	1.7430	1.33E-02
243546_at	---	1.7354	1.68E-02
235213_at	ITPKB	1.6422	3.67E-02
230536_at	PBX4	1.5972	1.37E-02
230233_at	---	1.5334	3.35E-04
244026_at	---	1.4736	3.30E-02
239301_at	---	1.4725	2.20E-02
205590_at	RASGRP1	1.4560	5.14E-03
201464_x_at	JUN	1.4555	7.01E-03
205027_s_at	MAP3K8	1.4488	3.87E-02
57540_at	RBKS	1.4379	1.40E-03
212841_s_at	PPFIBP2	1.4203	1.74E-02
213488_at	SNED1	1.4030	2.33E-02
228097_at	MYLIP	1.4018	4.11E-02

Probe set	Gene symbol	LogRatio	FDR
233899_x_at	ZBTB10	1.3954	9.93E-03
226039_at	MGAT4A	1.3927	2.99E-02
209626_s_at	OSBPL3	1.3832	1.37E-02
219734_at	SIDT1	1.3802	2.43E-02
226142_at	GLIPR1	1.3731	2.88E-03
214657_s_at	NEAT1	1.3647	2.03E-02
227062_at	---	1.3613	4.00E-02
201422_at	IFI30	1.3528	2.56E-02
228528_at	LOC100286909	1.3480	3.75E-03
219312_s_at	ZBTB10	1.3296	9.52E-03
36711_at	MAFF	1.2934	3.74E-02
211458_s_at	GABARAPL1 /// GABARAPL3	1.2903	6.35E-03
243918_at	---	1.2882	1.12E-02
224523_s_at	C3orf26	1.2852	3.52E-02
244349_at	---	1.2797	4.31E-02
214470_at	KLRB1	1.2776	2.89E-02
212509_s_at	MXRA7	1.2637	3.48E-04
202466_at	PAPD7	1.2628	2.15E-03
241985_at	JMY	1.2511	5.12E-03
204897_at	PTGER4	1.2358	2.03E-02
1559060_a_at	FNIP1	1.2158	2.49E-02
207072_at	IL18RAP	1.2137	1.73E-02
1555827_at	CCNL1	1.2096	8.07E-03
212796_s_at	TBC1D2B	1.2039	1.15E-03
218845_at	DUSP22	1.2011	1.65E-02
204222_s_at	GLIPR1	1.2005	2.07E-03
1553133_at	C9orf72	1.1962	4.71E-02
210606_x_at	KLRD1	1.1870	2.54E-02
225651_at	UBE2E2	1.1829	1.13E-02
214085_x_at	GLIPR1	1.1588	2.10E-02
226136_at	GLIPR1	1.1575	3.99E-03
232929_at	---	1.1469	3.59E-02
214446_at	ELL2	1.1454	1.50E-02
211423_s_at	SC5DL	1.1425	1.89E-02
1554690_a_at	TACC1	1.1370	2.19E-02
210136_at	MBP	1.1357	2.87E-02
204896_s_at	PTGER4	1.1267	1.19E-02
212636_at	QKI	1.1109	3.11E-02
226099_at	ELL2	1.1085	1.37E-02
212676_at	NF1	1.1033	1.80E-03
215013_s_at	USP34	1.1023	5.84E-03
228167_at	KLHL6	1.0956	4.58E-03
1554544_a_at	MBP	1.0800	3.31E-02
224250_s_at	SECISBP2	1.0772	4.38E-02
218329_at	PRDM4	1.0729	2.80E-04
210077_s_at	SFRS5	1.0719	3.94E-02
1566958_at	---	1.0717	2.05E-02
236139_at	---	1.0670	5.56E-03
204221_x_at	GLIPR1	1.0655	3.32E-02
1566959_at	---	1.0579	3.33E-02
213793_s_at	HOMER1	1.0573	1.54E-02
239978_at	---	1.0569	4.22E-02
226982_at	ELL2	1.0536	2.92E-02

Appendixes

Probe set	Gene symbol	LogRatio	FDR
227478_at	SETBP1	1.0491	2.85E-02
201751_at	JOSD1	1.0489	2.50E-04
208869_s_at	GABARAPL1	1.0481	2.22E-02
204821_at	BTN3A3	1.0461	2.78E-02
204137_at	GPR137B	1.0445	2.28E-02
219506_at	C1orf54	1.0418	1.07E-04
201465_s_at	JUN	1.0415	1.92E-02
213281_at	LOC100288387	1.0400	2.30E-02
221986_s_at	KLHL24	1.0394	1.63E-02
226440_at	DUSP22	1.0385	3.03E-02
222848_at	CENPK	1.0338	1.70E-02
49485_at	PRDM4	1.0278	4.84E-04
239912_at	---	1.0250	1.79E-03
227626_at	PAQR8	1.0231	1.46E-02
228967_at	EIF1	1.0143	1.14E-02
239845_at	---	1.0120	5.68E-04
225522_at	AAK1	1.0048	2.65E-02
239163_at	UBE2B	1.0014	3.49E-02
232134_at	---	1.0008	2.02E-02
201300_s_at	PRNP	0.9967	4.62E-02
214814_at	YTHDC1	0.9896	2.33E-02
226352_at	JMY	0.9893	7.09E-04
235536_at	SNORD89	0.9871	2.38E-04
206966_s_at	KLF12	0.9816	2.37E-02
1562280_at	---	0.9794	1.95E-02
209257_s_at	SMC3	0.9746	1.64E-02
225919_s_at	C9orf72	0.9735	9.88E-03
230302_at	---	0.9617	2.87E-02
219696_at	DENND1B	0.9525	3.36E-02
201531_at	ZFP36	0.9490	4.28E-03
224566_at	NEAT1	0.9483	1.49E-02
233952_s_at	ZNF295	0.9417	1.24E-03
239331_at	---	0.9407	1.45E-02
219222_at	RBKS	0.9384	3.57E-03
227100_at	B3GALT1	0.9383	9.00E-03
244341_at	---	0.9325	2.32E-02
212658_at	LHFPL2	0.9315	3.96E-02
202814_s_at	HEXIM1	0.9302	4.00E-02
229312_s_at	GKAP1	0.9229	2.45E-02
226026_at	DIRC2	0.9197	2.23E-02
210656_at	EED	0.9190	1.50E-02
228768_at	FNIP1	0.9181	1.97E-04
214696_at	C17orf91	0.9159	2.89E-02
205251_at	PER2	0.9145	3.33E-02
202364_at	MXI1	0.9132	1.91E-03
206917_at	GNA13	0.9126	5.84E-03
230494_at	---	0.9117	4.13E-02
202265_at	BMI1	0.9052	2.64E-03
1559119_at	---	0.9008	4.12E-02
227539_at	GNA13	0.8972	6.78E-03
228774_at	CEP78	0.8917	3.56E-03
242647_at	USP34	0.8916	9.59E-03
229699_at	LOC100129550	0.8902	4.39E-02

Probe set	Gene symbol	LogRatio	FDR
228603_at	ACTR3	0.8883	3.20E-02
218196_at	OSTM1	0.8858	9.56E-03
239405_at	---	0.8838	4.91E-02
217437_s_at	TACC1	0.8837	3.61E-03
225553_at	---	0.8814	2.03E-02
226679_at	SLC26A11	0.8693	1.48E-02
209383_at	DDIT3	0.8678	5.55E-03
207920_x_at	ZFX	0.8660	1.59E-02
203243_s_at	PDLIM5	0.8623	2.95E-02
242287_at	CLIP1	0.8583	3.91E-03
221985_at	KLHL24	0.8581	1.02E-02
227562_at	MAPKSP1	0.8533	3.41E-03
218772_x_at	TMEM38B	0.8512	6.78E-03
214429_at	MTMR6	0.8508	9.93E-03
224601_at	---	0.8479	4.50E-02
208744_x_at	HSPH1	0.8477	2.44E-02
1556121_at	NAP1L1	0.8464	9.59E-03
210793_s_at	NUP98	0.8463	1.07E-02
1558407_at	PLEKHG2	0.8442	1.19E-02
239130_at	MIR101-1	0.8428	5.14E-04
1555785_a_at	XRN1	0.8423	3.96E-02
203723_at	ITPKB	0.8417	3.86E-02
1555790_a_at	TMEM192 /// ZNF320	0.8403	7.19E-04
218349_s_at	ZWILCH	0.8329	2.28E-02
238707_at	---	0.8268	1.44E-02
212926_at	SMC5	0.8255	2.89E-02
226989_at	RGMB	0.8253	1.85E-02
200783_s_at	STMN1	0.8232	8.08E-03
212119_at	RHOQ	0.8228	3.59E-02
223513_at	CENPJ	0.8211	9.27E-04
224978_s_at	USP36	0.8187	3.88E-02
202899_s_at	SFRS3	0.8162	1.51E-02
202815_s_at	HEXIM1	0.8150	3.24E-02
227433_at	KIAA2018	0.8146	8.58E-05
226589_at	TMEM192	0.8091	1.10E-02
228433_at	NFYA	0.8087	5.65E-03
202255_s_at	SIPA1L1	0.8082	1.33E-02
221808_at	RAB9A	0.8045	7.21E-03
227337_at	ANKRD37	0.8045	4.74E-02
226651_at	HOMER1	0.8003	4.44E-02
208994_s_at	PPIG	0.7980	3.46E-02
235980_at	PIK3CA	0.7958	9.53E-03
1553719_s_at	ZNF548	0.7932	6.23E-03
1569380_a_at	---	0.7893	1.87E-03
212218_s_at	FASN	0.7874	2.26E-02
224956_at	NUFIP2	0.7873	1.42E-03
239826_at	---	0.7868	4.71E-02
1552611_a_at	JAK1	0.7860	2.40E-02
243404_at	---	0.7856	4.57E-02
213379_at	COQ2	0.7819	1.70E-02
228562_at	ZBTB10	0.7810	4.15E-02
234594_at	NCRNA00203	0.7786	3.83E-02
226423_at	PAQR8	0.7753	5.65E-03

Probe set	Gene symbol	LogRatio	FDR
222735_at	TMEM38B	0.7746	2.32E-02
1555247_a_at	RAPGEF6	0.7724	8.42E-03
213434_at	STX2	0.7711	1.49E-02
235919_at	---	0.7707	1.17E-02
242290_at	TACC1	0.7698	7.19E-03
201471_s_at	SQSTM1	0.7632	1.14E-02
210818_s_at	BACH1	0.7632	3.32E-02
231199_at	---	0.7618	6.47E-03
235198_at	OSTM1	0.7606	3.50E-02
223376_s_at	BRI3	0.7605	2.03E-02
208673_s_at	SFRS3	0.7588	4.41E-04
231975_s_at	MIER3	0.7570	8.84E-03
206976_s_at	HSPH1	0.7552	3.37E-02
218648_at	CRTC3	0.7514	4.57E-02

4.2. Probe sets with higher expression in the treated cells of the high ZAP70 group

Probe set	Gene symbol	LogRatio	FDR
204731_at	TGFBR3	3.6210	6.34E-04
233483_at	TBC1D27	3.5233	1.65E-05
226625_at	TGFBR3	3.3333	9.01E-05
205204_at	NMB	2.8912	1.80E-03
209732_at	CLEC2B	2.5529	3.11E-03
214836_x_at	IGK@ /// IGKC	2.4450	3.64E-02
206760_s_at	FCER2	2.0422	1.44E-02
226517_at	BCAT1	2.0379	1.07E-02
214916_x_at	IGH@ /// IGHA1	1.9926	2.31E-02
205081_at	CRIP1	1.9883	1.06E-03
242572_at	---	1.9788	4.65E-02
225285_at	BCAT1	1.9777	1.22E-02
207761_s_at	METTL7A	1.9628	3.48E-04
206759_at	FCER2	1.9505	1.43E-02
204135_at	FILIP1L	1.9413	5.56E-03
213839_at	CLMN	1.8813	2.18E-04
202283_at	SERPINF1	1.8703	1.28E-02
212504_at	DIP2C	1.8687	5.45E-03
203186_s_at	S100A4	1.8664	3.80E-03
238071_at	LCN10 /// LCN6	1.7831	3.24E-03
238559_at	---	1.7778	4.53E-04
202096_s_at	TSPO	1.7777	1.46E-02
203508_at	TNFRSF1B	1.7331	1.75E-02
1554966_a_at	FILIP1L	1.7311	8.83E-03
219256_s_at	SH3TC1	1.7117	3.49E-02
203921_at	CHST2	1.7108	2.01E-05
225382_at	ZNF275	1.7106	1.47E-04
201850_at	CAPG	1.6997	7.83E-05
205462_s_at	HPCAL1	1.6902	1.24E-03
1555613_a_at	ZAP70	1.6856	2.62E-03
229437_at	MIR155HG	1.6524	1.44E-03
205353_s_at	PEBP1	1.6277	1.31E-04
218100_s_at	IFT57	1.6212	3.50E-05

Probe set	Gene symbol	LogRatio	FDR
226358_at	APH1B	1.6202	6.33E-05
230363_s_at	INPP5F	1.6046	8.82E-03
201037_at	PFKP	1.5906	1.07E-04
203711_s_at	HIBCH	1.5830	2.82E-03
205547_s_at	TAGLN	1.5752	1.97E-04
235372_at	FCRLA	1.5703	7.43E-04
224990_at	C4orf34	1.5669	2.80E-03
228056_s_at	NAPSB	1.5436	3.42E-03
206150_at	CD27	1.5423	1.24E-03
211941_s_at	PEBP1	1.5321	1.83E-04
214366_s_at	ALOX5	1.5283	7.32E-04
214238_at	---	1.5157	1.16E-05
225757_s_at	CLMN	1.5072	1.09E-04
214032_at	ZAP70	1.5044	2.62E-03
239893_at	---	1.5014	2.50E-02
208614_s_at	FLNB	1.4824	2.43E-03
221036_s_at	APH1B	1.4750	4.57E-06
204401_at	KCNN4	1.4500	2.50E-04
232773_at	---	1.4456	3.10E-03
210609_s_at	TP53I3	1.4435	8.33E-04
210825_s_at	PEBP1	1.4417	1.46E-04
205267_at	POU2AF1	1.4414	3.24E-03
1565754_x_at	FGD2	1.4354	1.31E-04
225164_s_at	EIF2AK4	1.4351	2.64E-03
237400_at	ATP5S	1.4347	7.18E-03
210448_s_at	P2RX5	1.4294	2.71E-04
216620_s_at	ARHGEF10	1.4185	2.14E-02
225624_at	SNX29	1.4068	1.07E-04
219173_at	MYO15B	1.3978	1.03E-02
1555724_s_at	TAGLN	1.3940	6.33E-05
229114_at	GAB1	1.3844	3.11E-03
226002_at	GAB1	1.3838	6.32E-03
222519_s_at	IFT57	1.3835	2.95E-04
228055_at	NAPSB	1.3829	7.54E-03
220016_at	AHNAK	1.3823	2.91E-03
59375_at	MYO15B	1.3756	6.26E-03
235459_at	---	1.3730	1.36E-03
1555379_at	FAM159A	1.3704	2.70E-02
218280_x_at	HIST2H2AA3 /// HIST2H2AA4	1.3660	8.21E-03
208997_s_at	UCP2	1.3623	3.42E-03
211430_s_at	IGH@ /// IGHG1	1.3613	4.59E-02
206255_at	BLK	1.3506	1.33E-02
1565752_at	FGD2	1.3474	2.16E-04
223991_s_at	GALNT2 /// LOC100132910	1.3440	3.50E-02
207641_at	TNFRSF13B	1.3421	1.85E-03
201028_s_at	CD99	1.3420	5.65E-03
244050_at	PTPLAD2	1.3358	3.66E-03
214290_s_at	HIST2H2AA3 /// HIST2H2AA4	1.3269	3.14E-02
230362_at	INPP5F	1.3205	1.50E-02
230381_at	C1orf186	1.3183	2.04E-02
219518_s_at	ELL3 /// SERINC4	1.3160	3.43E-03
213374_x_at	HIBCH	1.3136	1.00E-02
230983_at	FAM129C	1.2911	2.27E-02

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Probe set	Gene symbol	LogRatio	FDR
1552667_a_at	SH2D3C	1.2891	2.94E-03
227052_at	---	1.2786	4.75E-03
212552_at	HPCAL1	1.2781	2.31E-03
210785_s_at	C1orf38	1.2767	1.24E-02
224989_at	---	1.2637	2.92E-02
230131_x_at	ARSD	1.2634	6.77E-03
226147_s_at	PIGR	1.2583	2.78E-02
205677_s_at	DLEU1	1.2529	2.56E-02
207571_x_at	C1orf38	1.2430	9.93E-03
205297_s_at	CD79B	1.2429	5.65E-03
225383_at	ZNF275	1.2361	2.62E-03
204257_at	FADS3	1.2265	1.75E-02
1553906_s_at	FGD2	1.2191	2.42E-03
238376_at	---	1.2182	3.82E-02
236539_at	PTPN22	1.2131	1.75E-02
227607_at	STAMBPL1	1.2070	2.38E-04
208613_s_at	FLNB	1.2027	4.58E-03
1552623_at	HSH2D	1.1962	9.83E-04
229597_s_at	WDFY4	1.1872	2.42E-03
229021_at	MCTP2	1.1857	3.20E-02
224735_at	CYBASC3	1.1827	1.31E-04
214475_x_at	CAPN3	1.1812	2.80E-03
203607_at	INPP5F	1.1718	9.52E-03
201760_s_at	WSB2	1.1676	8.42E-03
204445_s_at	ALOX5	1.1562	2.62E-03
236016_at	---	1.1526	2.71E-02
208998_at	UCP2	1.1513	8.58E-04
237033_at	FAM159A	1.1438	2.51E-04
228532_at	C1orf162	1.1433	3.03E-02
219282_s_at	TRPV2	1.1361	2.04E-02
242722_at	LMO7	1.1327	9.56E-03
200736_s_at	GPX1	1.1241	1.03E-02
211986_at	AHNAK	1.1227	5.73E-03
228614_at	NCRNA00116	1.1197	1.33E-02
234050_at	TAGAP	1.1160	2.21E-02
1553102_a_at	CCDC69	1.1141	1.02E-03
202942_at	ETFB	1.1121	2.39E-04
226134_s_at	---	1.1108	4.56E-02
201962_s_at	RNF41	1.1094	3.66E-03
226673_at	SH2D3C	1.1091	1.40E-03
37965_at	PARVB	1.0988	2.82E-03
228518_at	IGH@ /// IGHG1 /// IGHM	1.0955	2.33E-02
212611_at	DTX4	1.0949	1.21E-02
227606_s_at	STAMBPL1	1.0912	2.89E-03
206060_s_at	PTPN22	1.0904	1.13E-02
211771_s_at	POU2F2	1.0887	3.50E-02
209703_x_at	METTL7A	1.0882	1.33E-03
225998_at	GAB1	1.0850	2.16E-02
37966_at	PARVB	1.0833	8.88E-03
224512_s_at	LSMD1	1.0829	1.21E-04
201961_s_at	RNF41	1.0794	1.13E-02
209806_at	HIST1H2BK	1.0778	2.56E-02
244172_at	---	1.0757	4.01E-02

Probe set	Gene symbol	LogRatio	FDR
1555779_a_at	CD79A	1.0693	5.14E-04
224856_at	FKBP5	1.0678	4.17E-02
224789_at	DCAF12	1.0661	2.65E-03
211890_x_at	CAPN3	1.0632	9.48E-04
216958_s_at	IVD	1.0630	3.61E-03
203523_at	LSP1	1.0581	1.70E-02
225637_at	DEF8	1.0574	1.33E-02
221666_s_at	PYCARD	1.0565	1.80E-02
210051_at	RAPGEF3	1.0512	2.44E-02
229958_at	CLN8	1.0495	3.02E-02
227182_at	SUSD3	1.0491	3.80E-03
227134_at	SYTL1	1.0441	2.94E-03
1553857_at	IGSF22	1.0417	2.54E-02
219517_at	ELL3 /// SERINC4	1.0403	6.67E-03
218231_at	NAGK	1.0394	4.32E-03
234759_at	---	1.0359	1.07E-02
206687_s_at	PTPN6	1.0333	2.11E-02
205049_s_at	CD79A	1.0297	2.38E-04
205901_at	PNOC	1.0211	2.83E-04
226622_at	MUC20	1.0178	1.23E-02
207819_s_at	ABCB4	1.0109	4.53E-02
227943_at	---	1.0107	1.65E-02
201828_x_at	FAM127A	1.0097	1.83E-04
225723_at	C6orf129	1.0076	5.14E-03
214084_x_at	NCF1C	1.0022	1.60E-03
200707_at	PRKCSH	1.0009	6.67E-03
224666_at	NSMCE1	1.0003	1.79E-03
221286_s_at	MGC29506	0.9983	4.39E-02
239233_at	CCDC88A	0.9941	4.39E-02
1570505_at	ABCB4	0.9931	2.13E-02
204961_s_at	NCF1 /// NCF1B /// NCF1C	0.9899	2.80E-03
223553_s_at	DOK3	0.9869	4.38E-03
209916_at	DHTKD1	0.9859	1.32E-02
225608_at	SNX29	0.9844	6.33E-05
204446_s_at	ALOX5	0.9802	8.22E-03
208010_s_at	PTPN22	0.9782	4.60E-02
201029_s_at	CD99	0.9762	2.88E-03
221807_s_at	TRABD	0.9760	2.24E-02
212886_at	CCDC69	0.9748	7.32E-04
236496_at	DEGS2	0.9739	1.50E-02
200678_x_at	GRN	0.9658	2.56E-02
209582_s_at	CD200	0.9647	1.92E-02
217478_s_at	HLA-DMA	0.9633	2.83E-04
207785_s_at	RBPJ	0.9611	2.23E-02
214354_x_at	SFTPB	0.9609	4.38E-02
1554574_a_at	CYB5R3	0.9576	2.33E-02
202587_s_at	AK1	0.9559	9.59E-03
201769_at	CLINT1	0.9508	1.16E-05
243699_at	---	0.9499	1.01E-02
211991_s_at	HLA-DPA1	0.9494	2.75E-03
223514_at	CARD11	0.9485	1.80E-03
205718_at	ITGB7	0.9471	1.75E-02
226419_s_at	FLJ44342	0.9464	4.28E-02

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Probe set	Gene symbol	LogRatio	FDR
218312_s_at	ZSCAN18	0.9425	4.11E-02
1558345_a_at	LOC439911	0.9413	1.80E-02
214907_at	CEACAM21	0.9395	2.24E-02
216041_x_at	GRN	0.9376	3.13E-02
225441_x_at	LSMD1	0.9374	2.00E-05
203233_at	IL4R	0.9355	4.37E-03
205801_s_at	RASGRP3	0.9352	8.42E-03
221551_x_at	ST6GALNAC4	0.9345	2.24E-02
203454_s_at	ATOX1	0.9343	1.12E-02
229750_at	POU2F2	0.9340	1.34E-02
217728_at	S100A6	0.9309	4.21E-02
212167_s_at	SMARCB1	0.9289	9.40E-03
209583_s_at	CD200	0.9282	1.91E-02
225311_at	IVD	0.9274	1.33E-02
219279_at	DOCK10	0.9266	9.44E-03
205213_at	ACAP1	0.9264	9.59E-03
226856_at	MUSTN1	0.9242	6.10E-03
222245_s_at	FER1L4	0.9218	5.59E-03
225294_s_at	TRAPPC1	0.9166	3.56E-03
204929_s_at	VAMP5	0.9120	2.24E-02
226482_s_at	TSTD1	0.9119	4.99E-02
229050_s_at	SNHG7	0.9116	5.09E-03
207655_s_at	BLNK	0.9074	3.25E-02
210621_s_at	RASA1	0.9069	1.92E-02
229681_at	---	0.9025	1.24E-03
232344_at	---	0.8989	1.47E-02
209441_at	RHOBTB2	0.8983	2.82E-03
225700_at	GLCCI1	0.8977	1.96E-02
205861_at	SPIB	0.8973	3.96E-02
208690_s_at	PDLIM1	0.8958	7.19E-04
202732_at	PKIG	0.8956	2.95E-02
221011_s_at	LBH	0.8952	2.89E-02
223740_at	C6orf59	0.8952	2.20E-02
218164_at	SPATA20	0.8927	4.61E-02
230888_at	WDR91	0.8912	1.13E-02
209143_s_at	CLNS1A	0.8903	7.01E-03
1568658_at	C2orf74	0.8896	2.97E-02
1558105_a_at	---	0.8887	2.36E-02
228264_at	ACCS	0.8825	3.86E-02
233167_at	SELO	0.8785	4.57E-02
223299_at	SEC11C	0.8753	7.09E-04
210889_s_at	FCGR2B	0.8749	4.57E-02
210944_s_at	CAPN3	0.8745	4.86E-02
223686_at	TPK1	0.8743	1.69E-02
229130_at	---	0.8730	2.54E-02
211284_s_at	GRN	0.8715	3.14E-02
238894_at	---	0.8692	4.44E-02
212350_at	TBC1D1	0.8668	1.78E-02
224482_s_at	RAB11FIP4	0.8622	1.21E-02
228008_at	---	0.8612	1.89E-02
214995_s_at	APOBEC3F /// APOBEC3G	0.8597	4.87E-02
204513_s_at	ELMO1	0.8567	1.85E-02
214339_s_at	MAP4K1	0.8547	6.01E-03

Probe set	Gene symbol	LogRatio	FDR
213600_at	SIPA1L3	0.8534	3.43E-03
201486_at	RCN2	0.8519	1.25E-02
213170_at	GPX7	0.8513	1.46E-02
205423_at	AP1B1	0.8477	3.80E-03
203421_at	TP53I11	0.8471	3.70E-03
227525_at	GLCCI1	0.8463	1.07E-02
208714_at	NDUFV1	0.8449	1.33E-03
222859_s_at	DAPP1	0.8367	1.33E-02
206398_s_at	CD19	0.8364	4.17E-03
224715_at	WDR34	0.8363	4.20E-03
221058_s_at	CKLF	0.8312	3.43E-02
237367_x_at	CFLAR	0.8302	2.99E-05
203167_at	TIMP2	0.8289	3.76E-02
203236_s_at	LGALS9	0.8288	3.48E-02
228943_at	MAP6	0.8288	4.42E-02
227414_at	RHBDD1	0.8248	2.91E-03
206296_x_at	MAP4K1	0.8229	1.45E-02
210184_at	ITGAX	0.8185	2.80E-02
236198_at	---	0.8130	2.99E-02
205484_at	SIT1	0.8102	1.12E-02
205180_s_at	ADAM8	0.8089	2.70E-02
222238_s_at	POLM	0.8088	1.74E-02
211075_s_at	CD47	0.8073	4.74E-04
201825_s_at	SCCPDH	0.8061	2.46E-02
201487_at	CTSC	0.8046	6.35E-03
229892_at	EP400NL	0.8031	1.31E-02
235512_at	CDKL1	0.8028	2.27E-02
215688_at	RASGRF1	0.8024	4.30E-02
244220_at	---	0.8022	3.57E-03
219032_x_at	OPN3	0.8020	2.92E-03
203555_at	PTPN18	0.7995	2.31E-02
209509_s_at	DPAGT1	0.7989	9.43E-03
238920_at	---	0.7978	4.32E-02
227321_at	GATS	0.7978	4.37E-03
226948_at	RHBDD1	0.7969	3.75E-03
204483_at	ENO3	0.7969	3.66E-02
212778_at	PACS2	0.7962	5.09E-03
227947_at	PHACTR2	0.7950	2.49E-02
228956_at	UGT8	0.7949	5.76E-03
224821_at	ABHD14B	0.7938	3.37E-02
225175_s_at	SLC44A2	0.7932	8.50E-03
235427_at	---	0.7927	2.01E-02
211299_s_at	FLOT2	0.7911	8.11E-03
202253_s_at	DNM2	0.7900	5.27E-03
228994_at	CCDC24	0.7886	6.67E-03
227749_at	---	0.7877	2.56E-02
226608_at	C16orf87	0.7873	2.03E-02
242556_at	---	0.7868	1.53E-02
226795_at	LRCH1	0.7840	1.39E-02
216253_s_at	PARVB	0.7840	2.41E-02
1554452_a_at	C7orf68	0.7824	2.30E-02
211656_x_at	HLA-DQB1 /// LOC100294318	0.7822	4.61E-03
203358_s_at	EZH2	0.7820	3.80E-02

Appendixes

Probe set	Gene symbol	LogRatio	FDR
209166_s_at	MAN2B1	0.7789	8.42E-03
217948_at	FAM127B	0.7773	5.91E-03
204205_at	APOBEC3G	0.7760	3.43E-02
214219_x_at	MAP4K1	0.7728	1.40E-02
206287_s_at	ITIH4	0.7724	4.23E-03
212935_at	MCF2L	0.7716	2.31E-02
229707_at	ZNF606	0.7688	2.80E-03
201350_at	FLOT2	0.7652	1.50E-03
221725_at	WASF2	0.7651	1.13E-02
201216_at	ERP29	0.7636	3.91E-03
224562_at	WASF2	0.7590	2.39E-02
202208_s_at	ARL4C	0.7538	2.03E-02
226878_at	HLA-DOA	0.7515	2.95E-02

APPENDIX 5

5.1. Manuscript published in Clinical Cancer Research:

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Differential Gene Expression Profile Associated to Apoptosis Induced by Dexamethasone in CLL Cells According to IGHV/ZAP-70 Status

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Running title:

CLL response to dexamethasone according to IGHV/ZAP-70

Keywords:

CLL; dexamethasone; GEP; FKBP5; GILZ

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Translational relevance

Some patients with chronic lymphocytic leukemia (CLL) are refractory to conventional treatments and in this setting one of the therapeutic options are the glucocorticoids. Herein we provide the first 'gene/molecular fingerprint' of dexamethasone in CLL cells. Our results corroborate the better response to glucocorticoids of CLL cells from patients from the poor outcome subgroup with unmutated IGHV genes/high ZAP-70 expression and describe some genes associated to this differential response.

The better understanding of the effect of dexamethasone in CLL cells can unveil new therapeutic targets for chemotherapy combinations and can facilitate the development of predictive markers of response to this drug.

ABSTRACT**Purpose**

Glucocorticoids are part of the therapeutic armamentarium of chronic lymphocytic leukemia (CLL) where it has been suggested that cells with unmutated IGHV genes exhibit higher sensitivity. The mechanisms by which glucocorticoids are active in CLL are not well elucidated. We aimed to ascertain the activity of dexamethasone in CLL cells according to prognosis and to identify the molecular mechanisms that are influencing the response to this drug.

Experimental design

Sensitivity to dexamethasone was analyzed *ex vivo* in 50 CLL and compared according to IGHV mutational status and/or ZAP-70 expression. The response was further compared by gene expression profiling (GEP) of selected cases. Expression of genes of interest was validated by quantitative reverse transcriptase PCR.

Results

Response to dexamethasone was higher in cases with unmutated IGHV/high ZAP-70 expression, and the levels of induction the pro-apoptotic Bim protein correlated with the degree of cell death. GEP analysis showed few genes differentially expressed after dexamethasone treatment between mutated and unmutated cases. However, functional annotation analysis showed that unmutated cases had significant enrichment in terms related to apoptosis. Specific analysis of genes of interest performed in a large series disclosed that in unmutated IGHV cells FKBP5 expression was higher at baseline and after

dexamethasone exposure, and that GILZ was more induced by dexamethasone treatment in these cases.

Conclusions

Unmutated IGHV/high ZAP-70 CLL cells exhibit better response to dexamethasone treatment, which is accompanied by a differential expression of genes involved in the glucocorticoid-receptor pathway and by an increased induction of genes related to apoptosis.

INTRODUCTION

Treatment of patients with chronic lymphocytic leukemia (CLL) has dramatically changed during the last decade with the introduction of monoclonal antibodies. Chemoimmunotherapy regimens like FCR (fludarabine, cyclophosphamide and rituximab)(1, 2), FCR plus mitoxantrone(3, 4), or FCR plus alemtuzumab(5) have proved to be highly effective in the treatment of this disease. Despite the excellent overall response and complete response rates obtained with these regimens, patients with 17p13.1 deletion and/or TP53 mutations usually exhibit a lower response rate, and shorter progression-free survival and overall survival (2, 6, 7). There is no standard salvage treatment for patients with refractory disease, particularly those with TP53 abnormalities, and therapeutic options are based on non-genotoxic drugs like alemtuzumab(8, 9), flavopiridol(10-12), lenalidomide(13, 14) or glucocorticoids(15-17), alone or in combination with monoclonal antibodies(5, 18-21). Patients that respond to these salvage treatments are recommended to undergo allogeneic stem-cell transplantation(22).

The mechanisms by which glucocorticoids induce CLL cell death are still not well understood. Glucocorticoids bind to a multiprotein complex receptor present in the cytoplasm constituted by the receptor itself and several cochaperones(23). After binding, the glucocorticoid-receptor dissociates from some of the cochaperone proteins and translocates into the nucleus, where it acts as a transcription factor modulating gene expression(24). Several studies have been conducted to identify genes that are regulated by glucocorticoids and have the ability to trigger lymphoid cell death, particularly in acute lymphoblastic

leukemia(25-30). In CLL, it has been found that dexamethasone up-regulates mRNA and protein expression of the pro-apoptotic BH3-only gene Bim(31). Of note, cell death induced by glucocorticoids is higher in CLL with unmutated IGHV genes (UCLL)/high ZAP-70 expression than in cases with mutated IGHV genes (MCLL)/low ZAP-70 (32-35), although the molecular mechanisms that could explain these differences have not been uncovered.

With this background, we aimed to widely analyze the modulation of gene expression induced by dexamethasone in CLL cells according to the different IGHV mutational status/ZAP-70 expression groups. For that, genome-wide gene expression profile (GEP) analysis was performed after dexamethasone treatment and genes of interest were retrieved for further study.

MATERIALS AND METHODS

Patient selection and sample collection

A group of 50 patients diagnosed with CLL was selected on the basis of the availability of frozen samples for biological studies. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, United Kingdom) density gradient and stored in liquid nitrogen until analysis. Informed consent from all patients was obtained according to the Declaration of Helsinki and the study was approved by the local clinical investigation ethical committee. The mean percentage of CLL cells (CD19+/CD5+ cells) in the series was 82.5% ± 9.7.

***Ex-vivo* treatment with dexamethasone and evaluation of the response**

PBMCs from CLL patients were thawed at 37°C and resuspended in standard culture medium (RPMI-1640 medium (Gibco, Paisley, Scotland, UK) supplemented with 10% heat-inactivated FBS (Gibco), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Lonza, Viviers, Belgium), 2 mM L-glutamine and 1 mM sodium pyruvate (Gibco)) and cultured at 37°C in a 5% CO₂ atmosphere at a density of 1×10^6 cells/ml. PBMCs were allowed to recover from the thawing process for one hour before manipulation. Samples were split in two for control and incubation with the glucocorticoid dexamethasone (Merck KGaA, Darmstadt, Germany) at a concentration of 13.25 μ M based on previous reports of CLL treatment *ex-vivo* (36). After 24 hours, cell viability was evaluated by surface annexin V binding and propidium iodide staining assessed by flow cytometry (rh Annexin V/FITC kit, Bender MedSystems, Vienna, Austria). Cell viability was measured as the percentage of double-negative cells for annexin V and propidium iodide. Dexamethasone response was calculated as the percentage of live cells after treatment with dexamethasone relative to the percentage of live cells in the untreated cells (left with standard media).

ZAP-70 and IGHV mutational status analysis

Mutational status of the IGHV genes and ZAP-70 expression by flow cytometry were determined as previously described(37). Patients with more than 98% germline identity for IGHV genes were considered to be unmutated. CLL cases were considered to have high ZAP-70 expression when ZAP-70 was $\geq 20\%$ (37).

Quantitative reverse transcriptase PCR (QRT-PCR)

Total RNA was extracted with Trizol reagent (Invitrogen Life Technologies, Paisley, Scotland, UK) according to manufacturer instructions. For QRT-PCR analysis, complementary DNA was synthesized from 1µg RNA. Expression of Bim (BCL2-like 11 (apoptosis facilitator)), FKBP5 (FK506 binding protein 5) and GILZ (glucocorticoid-induced leucine zipper) was analyzed using pre-developed TAQMAN assays (Applied Biosystems, Foster City, CA): Hs00197982_m1, Hs01561001_m1 and Hs00608272_m1, respectively; and the ABI PRISM 7900 sequence detector instrument (Applied Biosystems). The comparative Ct method ($\Delta\Delta C_t$) for relative quantification of gene expression was used. β -Glucuronidase gene expression (GUSB, Applied Biosystems) was used as internal control, and mRNA-expression levels were given as arbitrary units (AU) referred to a commercial standard mRNA (Control RNA (Human), Applied Biosystems). Fold change (gene induction) was determined as the ratio between expression in dexamethasone treated cells and expression in untreated cells.

Microarray analysis

Total RNA (2 µg) was converted into biotin-labeled cRNA and further fragmented and hybridized to oligonucleotide Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix Inc, Santa Clara, CA). Expression measures were normalized and summarized using the frozen robust multiarray analysis (fRMA) methodology(38). Clustering and heatmaps were performed with the TM4 Software Suite(39). Gene expression data with log values lower than 5 were discarded. Differential expression analysis was carried out by a linear model

using empirical Bayes method to moderate the standard errors of the estimated log-Ratio changes with the limma package(40). The online tool David (41) was used for the functional annotation analysis using the BP_FAT category of Gene Ontology (GO). The GEP data has been deposited at the National Centre for Biotechnology Information's Gene Expression Omnibus (GEO ID: GSE33135).

RNA interference experiments

Small interference RNA (Dharmacon, Lafayette CO) targeting FKBP5, GILZ, non-targeting or rhodamine-labelled as positive control for transfection (mean at 48h 35%) was transfected to $5 \cdot 10^6$ primary CLL cells in 500 μ l RPMI-10%FBS. Briefly, 500nM of siRNA was mixed with 4.5 μ l of Hiperfect Transfection Reagent (Qiagen, Hilden, Germany) and added drop wise to the cells after 10 minutes incubation at room temperature. The cells were used 48 hours post-transfection only when viability was superior to 50% as analyzed by annexinV-PI staining by flow cytometry. Gene silencing efficiency was analyzed by QRT-PCR.

Statistical analysis

Comparisons between groups were done using the Mann-Whitney test. Correlations between measures were performed using a parametric linear regression model. For all comparisons, P-values were two-sided and the type I error was set at 5%. Statistical analyses were performed with the use of SPSS v18.0 software (IBM, Somers, NY) and GraphPad Prism v5.0 software (La Jolla, CA).

RESULTS

CLL cases with unmutated IGHV genes and/or high ZAP-70 expression show a higher response to dexamethasone *ex-vivo*

Samples from 50 patients diagnosed with CLL were treated *ex-vivo* with dexamethasone and the response was evaluated after 24 hours. The characteristics of the series are shown in Supplementary Table 1. Briefly, median age at diagnosis was 58 years (range, 30-82 years) and there was a male predominance (72%). ZAP-70 expression was considered high in 48% of the patients. IGHV mutational status was assessed in 47 cases, 23 of them (49%) being considered as UCLL. All the MCLL cases had low ZAP-70 expression, whereas only one UCLL case showed a low expression of ZAP-70. FISH analysis for the main CLL chromosomal abnormalities was performed in 48 out of 50 patients at the time the samples were obtained. According to the hierarchical model(6), 45.8% of the patients showed isolated 13q14.3 deletion, 10.4% 17p13.1 deletion, 10.4% trisomy 12, 4.2% 11q22.3 deletion and 29.2% presented no abnormality. After 24 hours of treatment with 13.25 μ M dexamethasone, the percentage of live cells relative to untreated cells ranged from 42% to 100%. Notably, UCLL cases (n=23) had a significantly better response to dexamethasone than MCLL cases (n=24) (mean of cell viability \pm SD: 68% \pm 14.0 vs 85% \pm 11.3; P<0.001; Figure 1A). In agreement, response to dexamethasone was also better in cases with high ZAP-70 expression (n=24) than in those with low ZAP-70 (n=26) (mean of cell viability \pm SD: 68% \pm 13.9 vs 85% \pm 11.0; P<0.001; Figure 1B). Remarkably, cases with 17p13.1 and 11q22.3 deletion (n=7) had a better response to dexamethasone than cases without

these high-risk genetic abnormalities (n=41) (mean of cell viability \pm SD: 64% \pm 16.2 vs 79% \pm 13.9; P=0.026). Of note, the only case with 17p13.1 deletion and low ZAP-70 expression disclosed a poor response to dexamethasone (Supplementary Table 1). Moreover, after excluding the cases with high-risk genetic abnormalities (17p13.1 and 11q22.3 deletions), ZAP-70 expression retained its predictive value for response to dexamethasone (mean of cell viability \pm SD: high ZAP-70 (n=17) 71% \pm 13.1 vs low ZAP-70 (n=24) 85% \pm 11.4; P=0.001). In absolute terms, the mean percentage of live cells after thawing in this series was 72% \pm 14, whereas after 24 hours of culture the mean viability was 56% \pm 15 for control cells and 44% \pm 16 for treated cells.

Induction of Bim expression by dexamethasone correlates with the extent of apoptosis in CLL cells

The expression of the pro-apoptotic BH3-only gene Bim has been reported to be induced by dexamethasone at both mRNA and protein level in different cellular models, including CLL cells (26, 31, 42-44). To study the kinetics of induction of Bim after treatment with dexamethasone, levels of Bim mRNA were analyzed by QRT-PCR at different time points in primary cells from 7 patients with CLL. As early as after 3 hours of treatment, an increase in Bim mRNA was already detected; in five of the cases, levels kept increasing up to 9 hours and then remained stable, whereas in the other two cases an additional increase in Bim levels was observed from 9 to 24 hours (Figure 2A). Furthermore, the relationship between the magnitude of the response to dexamethasone and the degree of Bim induction was evaluated in 43 CLL samples after 24 hours of

treatment. An inverse correlation between Bim induction and the percentage of live cells was observed ($P=0.001$; Figure 2B). Moreover, levels of Bim induction were higher in the subgroup of CLL cases with high ZAP-70 expression ($n=19$) than in the subgroup with low ZAP-70 (mean Bim fold change \pm SD: 3.75 ± 1.89 vs 2.61 ± 0.78 ; $P=0.042$; Figure 2C) which is in agreement with the better response to dexamethasone observed in this subgroup. Altogether, these results indicate that Bim may be part of the apoptotic pathway triggered by dexamethasone.

Gene expression profiling of CLL samples treated with dexamethasone

GEP analysis was performed in a series of CLL patients to identify genes potentially implicated in the differential response to dexamethasone. For this, we selected 7 CLL samples with high ZAP-70 expression and 5 with low ZAP-70 expression (Supplementary Table 1). Tumor cells were treated with dexamethasone or left with standard media for 6 hours; this time point was selected on the basis that it preceded the highest levels of Bim induction observed after dexamethasone treatment (Figure 2A).

The unsupervised analysis of the expression data performed using the 1,000 probe sets showing the highest variability defined two main branches of samples according to ZAP-70 expression (Figure 3A).

The effect of dexamethasone treatment was then independently analyzed in the high and low ZAP-70 groups by means of supervised analysis considering only those changes in gene expression with a false discovery rate (FDR) value lower than 0.05 and a $\log\text{Ratio}>|0.75|$. We found that dexamethasone treatment up-regulated the expression of 314 probe sets (153 genes) in the group with high

ZAP-70 expression, whereas in the low ZAP-70 group a total of 226 probe sets (118 genes) resulted up-regulated (Supplementary Table 2). Among up-regulated genes, 190 probe sets were shared by both ZAP-70 expression groups. We conducted functional annotation analysis of genes differentially expressed using gene ontology (GO) categories for 'biological process'. This allows for the discovery of overrepresented categories of genes. Functional annotation analysis of up-regulated genes revealed that the most significant GO categories in the high and low ZAP-70 groups were related to apoptosis. Interestingly, the specific analysis of the common 190 probe sets showed that the most enriched category was *regulation of lymphoid activation*, which included genes such as IL7R and CTLA4. Of note, analysis of the 124 probe sets solely up-regulated in samples with high ZAP-70 expression showed a significant enrichment in genes involved in *positive regulation of apoptosis*, whereas analysis of the 36 probe sets only up-regulated in cases with low ZAP-70 disclosed that the most enriched GO category was related to *ion homeostasis*, a term that includes genes that participate in any process involved in the maintenance of an internal steady state of metal ions at the level of a cell, thus the relevance of apoptosis in this subgroup was less notorious. In conclusion, the enrichment in the apoptosis GO category observed in conjunctural analysis of high and low ZAP-70 groups was predominantly due to genes up-regulated only in cases with high ZAP-70 expression and better response to dexamethasone.

Dexamethasone treatment induced the down-regulation of 219 probe sets (153 genes) in CLL cases with high ZAP-70 expression and of 222 probe sets (155 genes) in cases with low ZAP-70 expression (Supplementary Table 2). Among

all down-regulated genes, a total of 132 probe sets were shared by both ZAP-70 groups. GO analysis of down-regulated probe sets showed that in both high and low ZAP-70 groups the most significant term was *immune response*. Of note, probe sets that were exclusively down-regulated in CLL cases with low ZAP-70 expression (n=90) were significantly enriched in genes related to *regulation of apoptosis*, being the majority of them involved in the positive regulation of this process.

The top 10 probe sets with the highest variation caused by the treatment with dexamethasone were subsequently selected (Table 1). Three genes were commonly up-regulated in high and low ZAP-70 groups, namely FKBP5, DDIT4 and TMEM2. In addition, 4 genes were commonly down-regulated by dexamethasone in both ZAP-70 expression groups: KMO, PALM2-AKAP2, IFIT2 and SAMD9L. Of note, FKBP5 was the most up-regulated gene in both ZAP-70 groups and was represented by three different probe sets. Interestingly, FKBP5 expression was also higher in the high ZAP-70 CLL group in both untreated (224840_at, logRatio=0.958, FDR=0.0129) and treated cells (probe sets: 224840_at, logRatio=0.734, FDR=0.0390 and 224856_at, logRatio=1.068, FDR=0.0416). The above mentioned results led us to hypothesize that the levels of FKBP5 could be involved in the differential response to dexamethasone observed in CLL cases.

Finally, we aimed to identify genes that had a significant differential regulation after treatment with dexamethasone in the two ZAP-70 expression groups. For this, the interaction term was calculated by assessing the difference between the genes induced/repressed by dexamethasone in the high ZAP-70 expression group and the genes induced/repressed by dexamethasone in the low ZAP-70

group. Considering P-values lower than 0.001, 45 probe sets (38 genes) were identified as differentially regulated (Figure 3B). GO analysis revealed a significant enrichment in genes related to regulation of apoptosis. Among these 38 differentially regulated genes we observed that TSC22D3 (alias GILZ) clustered with the pro-apoptotic gene BCL2L11 (alias Bim) (Figure 3B) indicating that the two genes were altered in a similar way by dexamethasone. In addition, only in the high ZAP-70 group, GILZ was one of the most up-regulated genes by dexamethasone (Table 1). These data suggested that GILZ may be implicated in the different response to dexamethasone observed in the ZAP-70 expression groups.

Increased levels of FKBP5 at baseline and after dexamethasone treatment correlate with enhanced apoptosis and high ZAP-70 expression

The GEP analysis revealed that FKBP5 was the most inducible gene by dexamethasone in CLL cells, its levels being higher in the high ZAP-70 subgroup. FKBP5 gene codifies for a cochaperone of the glucocorticoid-receptor complex which maintains the receptor complex in the cytoplasm. After glucocorticoid binding, FKBP5 is replaced by FKBP4 which allows for the nuclear translocation of the glucocorticoid-receptor complex(24). To further analyze the relationship between FKBP5 and the response to dexamethasone in CLL samples, we ascertained the expression of this gene by QRT-PCR in 46 CLL samples, 22 with high ZAP-70 expression, at baseline, at 6 hours after treatment with dexamethasone, and at 6 hours with media only. At baseline, levels of FKBP5 were higher in CLL cases with high ZAP-70 expression (n=16) than in those with low ZAP-70 (n=22) (mean levels of FKBP5 mRNA-expression

\pm SD: 0.95AU \pm 0.58 vs 0.57AU \pm 0.22; P=0.032; Figure 4A). FKBP5 baseline levels correlated with an increased apoptotic cell death at 24 hours of treatment with dexamethasone (n=38; P=0.027; Figure 4B). Interestingly, and in accordance with the GEP results, FKBP5 expression was induced 10 fold in mean after 6 hours of dexamethasone treatment (mean FKBP5 mRNA-expression \pm SD: treated cells (n=43) 3.04AU \pm 2.12 vs untreated cells (n=41) 0.36AU \pm 0.22; P<0.001; Figure 4C). Of note, levels of FKBP5 reached after 6 hours of treatment were significantly higher in cases with high ZAP-70 expression (n=20) than in those with low ZAP-70 (n=23) (mean FKBP5 mRNA-expression \pm SD: 4.31AU \pm 2.51 vs 1.95AU \pm 0.65; P<0.001; Figure 4D).

GILZ expression highly correlates with the induction of apoptosis by dexamethasone in CLL

GILZ is a transcription regulator directly targeted by the glucocorticoid-receptor (45) which negatively controls important mediators of cell proliferation (46). We found that GILZ was one of the top ten most inducible genes only in the high ZAP-70 group (Table 1). Moreover, GILZ was one of the few genes differently regulated by dexamethasone in the two ZAP-70 subgroups (Figure 3B).

To further assess the relationship between GILZ expression, ZAP-70 expression and the response to dexamethasone, levels of GILZ mRNA were determined by QRT-PCR in 40 CLL samples with or without treatment with dexamethasone for 6 hours. In untreated samples, levels of GILZ were higher in the low ZAP-70 group (n=20) than in the high ZAP-70 group (n=20) (mean GILZ mRNA-expression \pm SD: 40.45AU \pm 21.46 vs 32.71AU \pm 22.05; P=0.040; Figure 4E). Conversely, and according to GEP results, induction of GILZ after

treatment with dexamethasone was significantly higher in samples with high ZAP-70 expression (n=20) than in those with low ZAP-70 (n=20) (mean GILZ fold change \pm SD: 5.59 \pm 2.16 vs 3.92 \pm 0.83; P=0.002; Figure 4F). Moreover, we observed that this induction of GILZ correlated with cell viability (n=40; P<0.001; Figure 4G). Finally, and reinforcing that GILZ clustered with Bim in the GEP interaction term analysis, GILZ induction correlated with the increase of Bim expression (n=34; P=0.001; Figure 4H) determined after 24 hours of treatment with dexamethasone.

Inhibition of FKBP5 or GILZ expression by siRNA in primary CLL cells impairs response to dexamethasone treatment ex vivo.

In order to investigate if FKBP5 and GILZ are directly participating in the apoptotic response to dexamethasone observed in primary CLL cells, we analyzed the response to this treatment ex vivo after 48h of transfection with siRNA targeting FKBP5 or GILZ in four CLL cases. As can be observed in Figure 5, the percentage of live cells after 24 hours of treatment with 13.25 μ M dexamethasone is higher in CLL cells transfected with siRNA targeting FKBP5 or GILZ as compared to cells transfected with non-targeting RNA. The mean downregulation of FKBP5 was 26% and of GILZ 32%, as assessed by QRT-PCR, which led to a discrete but consistent decrease in the response to dexamethasone in all the cases analyzed. These results indicate that both FKBP5 and GILZ are indeed involved in the apoptotic response of CLL cells to dexamethasone ex vivo.

DISCUSSION

Herein we report that the degree of apoptosis induced by dexamethasone in neoplastic B CLL lymphocytes *ex-vivo* is significantly higher in patients with UCLL/high ZAP-70 expression than in patients with MCLL/low ZAP-70 expression, which have a better prognosis. This is in agreement with what has been previously described using prednisone and methylprednisolone(32-35). Interestingly, we showed that IGHV unmutated genes/high ZAP-70 expression conferred higher susceptibility to dexamethasone independently of the presence of 17p13.1 or 11q22.3 deletion. These results corroborated the clinical experience on the use of glucocorticoids in patients with high-risk cytogenetics (17, 19).

Induction of expression of Bim protein has been shown to be implicated in apoptosis induced by dexamethasone in ALL(26, 42-44) and this protein appeared to be the unique pro-apoptotic protein involved in cell death induced by glucocorticoids in CLL(31). In our study, besides confirming the early up-regulation of Bim expression on treatment with dexamethasone, we showed that dexamethasone-induced cell death positively correlated with levels of Bim induction. Altogether, these findings indicate that Bim is probably a downstream effector of dexamethasone in CLL. Since Bim pro-apoptotic mechanism has been demonstrated to be independent of p53 (47), its up-regulation could explain in part the response to glucocorticoids observed in some CLL cases with TP53 abnormalities(17, 19).

GEP analysis revealed high similarities between ZAP-70 subgroups in terms of genes regulated after dexamethasone treatment, indicating that the different

response to dexamethasone may not be due to an independent biological targeting of dexamethasone but to a differential capacity to induce cell death while inducing/repressing the same genes.

GEP results allowed us to select genes with significant levels of modulation along with biological relevance in the glucocorticoid pathway for further studies in larger series of patients. FKBP5, the cochaperone of the glucocorticoid-receptor(23), resulted to be the most inducible gene after dexamethasone treatment in both ZAP-70 subgroups. Moreover, we observed that baseline levels of expression of FKBP5 were higher in cases with high ZAP-70 expression by GEP and QRT-PCR experiments, the levels correlating with the extent of cell death. Interestingly, the downregulation of FKBP5 by siRNA decreased CLL cells sensitivity to dexamethasone. Our results are in line with previous studies performed in ALL, where the levels of glucocorticoid-receptor have been correlated with the degree of induced apoptosis(48). FKBP5 maintains the glucocorticoid-receptor in the cytoplasm in an active conformation(24), thus the higher levels of FKBP5 observed in cases of CLL with high ZAP-70 expression can be in part responsible for their better response to dexamethasone, however, in some cellular systems an overexpression of FKBP5 can actually reduce the transcriptional activity of the glucocorticoid-receptor, probably because of modification of the access of FKBP4 protein to the receptor, which allows nuclear translocation of the complex (24). Finally, GILZ, a previously know target of glucocorticoids (30, 49, 50) was identified in GEP analysis as differentially induced by dexamethasone, being higher in CLL samples with high ZAP-70 expression. Moreover, induction of GILZ was correlated with the induction of the downstream apoptotic effector Bim. GILZ

has been directly implicated in cell death after glucocorticoid treatment since its inhibition by siRNA impaired the apoptotic response in our and previous studies in multiple myeloma (49). Altogether these findings point toward a role of GILZ in apoptosis induced by glucocorticoids in CLL.

In summary, the induction of apoptosis by dexamethasone was higher in the cells from patients with UCLL/high ZAP-70 expression, being the induction of Bim positively correlated with the extent of apoptosis. The increased response to dexamethasone observed in cases with UCLL/high ZAP-70 expression is probably attributable to differences in baseline expression and induction of genes involved in the glucocorticoid and apoptosis pathways.

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TABLES

Table 1. The top 10 most up-regulated and down-regulated probe sets in CLL groups with high and low ZAP-70 expression after treatment with dexamethasone

ZAP-70 high				ZAP-70 low			
Gene Symbol	Probe set	logRatio	FDR	Gene Symbol	Probe set	logRatio	FDR
FKBP5*	224859_at	3.247	3.40E-07	FKBP5*	224840_at	2.917	9.51E-08
DDIT4*	202887_s_at	3.138	1.15E-07	CD72	215625_s_at	2.871	7.55E-08
FKBP5*	204590_at	3.073	7.48E-08	FKBP5*	224859_at	2.804	3.77E-05
TMEM2*	218113_at	2.942	3.22E-08	TMEM2*	218113_at	2.400	3.20E-08
TSC22D3	207001_x_at	2.760	9.40E-08	FKBP5*	204590_at	2.325	9.84E-04
FKBP5*	224840_at	2.693	1.94E-08	DHAP2	212936_at	2.229	9.15E-08
TGFBRS	228625_at	2.825	9.77E-08	HIPK3	225110_at	2.008	3.10E-05
TGFBRS	204731_at	2.408	1.24E-04	---	215528_at	2.078	3.31E-07
C18orf1	242551_at	2.384	7.79E-00	DDIT4*	202887_s_at	2.005	1.30E-04
---	242406_at	2.314	1.74E-08	---	241825_at	1.984	1.37E-04
down-regulated				down-regulated			
Gene Symbol	Probe set	logRatio	FDR	Gene Symbol	Probe set	logRatio	FDR
FCRL3	237093_at	-1.882	8.04E-08	CSL4	204103_at	-2.128	2.84E-08
KMO*	211138_s_at	-1.875	2.58E-07	AKAP2.//PALM-AKAP2*	202750_s_at	-1.728	6.48E-05
AKAP2.//PALM-AKAP2*	228604_at	-1.839	4.50E-08	AKAP2.//PALM-AKAP2*	228604_at	-1.800	4.13E-05
IFIT2*	228757_at	-1.828	3.15E-05	ISG15	205483_s_at	-1.537	1.22E-03
SETBP1	227478_at	-1.558	1.24E-05	SAMD1L*	228603_at	-1.519	1.01E-04
ANKRD2	222108_at	-1.543	5.93E-05	AKAP2.//PALM-AKAP2	202750_s_at	-1.494	7.21E-04
BCL2A1	205681_at	-1.639	8.90E-08	STAT1	AFFX-HUM00FSAM07935_MB_at	-1.408	1.81E-04
KMO	205308_x_at	-1.522	1.97E-07	KMO*	211138_s_at	-1.395	2.70E-05
SAMD1L*	228603_at	-1.403	1.03E-05	MIR21	224017_at	-1.392	1.90E-04
AKAP2.//PALM-AKAP2*	202750_s_at	-1.480	2.27E-05	IFIT2*	228757_at	-1.379	1.27E-08

Genes are ranked according to their logRatio values calculated as the difference in log expression value using the untreated cells group as baseline. *common probe sets in high and low ZAP-70 expression groups.

FIGURE LEGENDS

Figure 1. CLL cases with poor prognostic factors show better response to treatment with dexamethasone. CLL cells from 50 cases were treated with 13.25 μ M dexamethasone for 24 hours and the percentage of live cells was determined by annexinV/PI staining. (A) Response to dexamethasone in UCLL and MCLL. UCLL cases show significantly higher response to dexamethasone in terms of percentage of live cells than MCLL cases. (B) Response to dexamethasone in high and low ZAP-70 expression groups. CLL cases with high ZAP-70 expression have better response to dexamethasone than cases with low ZAP-70. Horizontal bars represent the mean values of live cells.

Figure 2. Bim is induced after treatment with dexamethasone and correlates with response and ZAP-70 expression. (A) Time-course of the induction of Bim after treatment with dexamethasone. CLL cells from 7 cases were treated with 13.25 μ M dexamethasone and Bim levels were evaluated at 3, 6, 9 and 24 hours by QRT-PCR. Results are expressed as the Bim fold change. HZ-CLL stands for high ZAP-70 expression and LZ-CLL for low ZAP-70 expression. The induction of Bim is high in the initial hours of treatment with dexamethasone and stabilizes after 9 hours. (B) Correlation of induction of Bim with the response to dexamethasone. CLL cells were treated with 13.25 μ M of dexamethasone for 24 hours and then both Bim fold change and response to treatment with dexamethasone were determined. The scatter-plot shows a linear correlation between induction of Bim and response to dexamethasone. (C) Induction of Bim in CLL cells with high or low ZAP-70 expression. CLL cells from 43 cases were

treated with 13.25 μM of dexamethasone for 24 hours and then both Bim fold change and response to dexamethasone treatment were determined. CLL cases with high ZAP-70 expression have significantly higher induction of Bim than cases with low ZAP-70. Horizontal bars represent the mean values of induction of Bim.

Figure 3. Gene expression profile analysis of CLL cells treated with dexamethasone according to ZAP-70 expression. (A) Dendrogram representing the unsupervised analysis of the 1,000 probe sets with the most variable expression applying the hierarchical clustering algorithm. DXM stands for dexamethasone treated cells and UNT stands for untreated cells; (B) Unsupervised cluster analysis of the 45 probe sets retrieved in the analysis of the interaction term. Changes in expression due to dexamethasone treatment for each probe set are displayed as LogRatios. HZ stands for high ZAP-70 expression and LZ stands for low ZAP-70 expression; in both (A) and (B) the number after CLL is the sample number according to Supplementary Table 1.

Figure 4. (A-D)FKBP5 levels at baseline and after treatment with dexamethasone correlate with higher response to dexamethasone and with ZAP-70 expression. CLL cells were treated with 13.25 μM of dexamethasone for 24 hours. Dexamethasone responses were determined at 24 hours. The levels of FKBP5 expression were determined by QRT-PCR. (A) At baseline, cases with high expression of ZAP-70 show higher levels of FKBP5 than cases with low ZAP-70. (B) Scatter-plot showing a significant negative correlation between the percentage of live cells after 24 hours of treatment with

dexamethasone and baseline FKBP5 levels. (C) After 6 hours, treated cells show higher levels of FKBP5 than untreated cells. (D) After 6 hours of treatment with dexamethasone, cases with high ZAP-70 expression show higher levels of FKBP5 than cases with low ZAP-70. In (A), (C) and (D) horizontal bars represent the mean value of the y-axis units.

(E-H) Induction of GILZ after 6 hours of treatment with dexamethasone correlates with the response to the treatment. The levels of expression of GILZ were determined by QRT-PCR after 6 hours of treatment. Fold change of Bim expression was determined by QRT-PCR at 24 hours. (E) The untreated cells from the cases with low expression of ZAP-70 show higher levels of GILZ mRNA than the cells from those with high ZAP-70. (F) After 6 hours of treatment with dexamethasone, cases with high ZAP-70 expression show higher induction of GILZ than cases with low ZAP-70. In (E) and (F) horizontal bars represent the mean value of the y-axis units. (G) Scatter-plot showing a significant negative correlation between GILZ induction and percentage of live cells after treatment with dexamethasone. (H) Scatter-plot showing a significant positive correlation between the induction of Bim and GILZ.

Figure 5. Inhibition of FKBP5 and GILZ by siRNA attenuates the response to dexamethasone. Primary CLL cells from 4 patients were transfected with siRNA targeting FKBP5, GILZ or with a non-targeting control. After 48 hours cells were treated with 13.25 μ M dexamethasone and the response was evaluated 24 hours later.

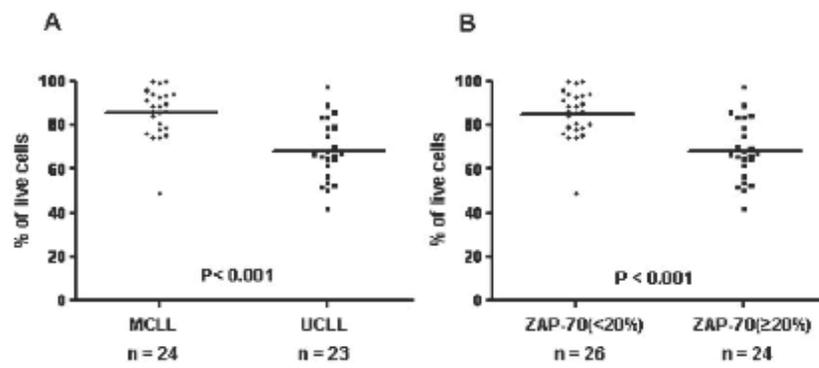


Figure 1

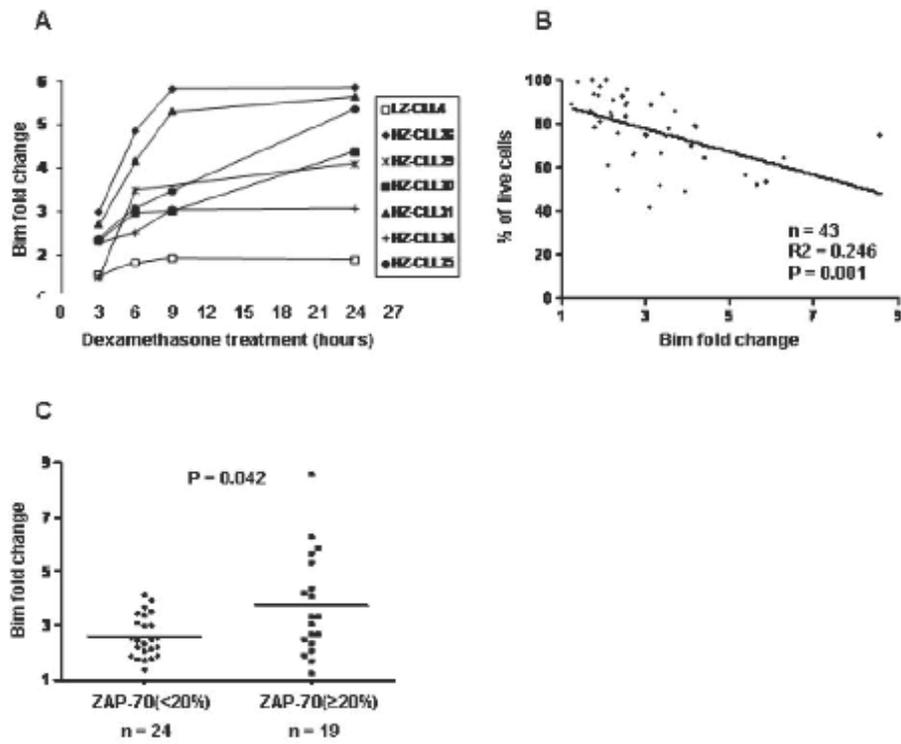
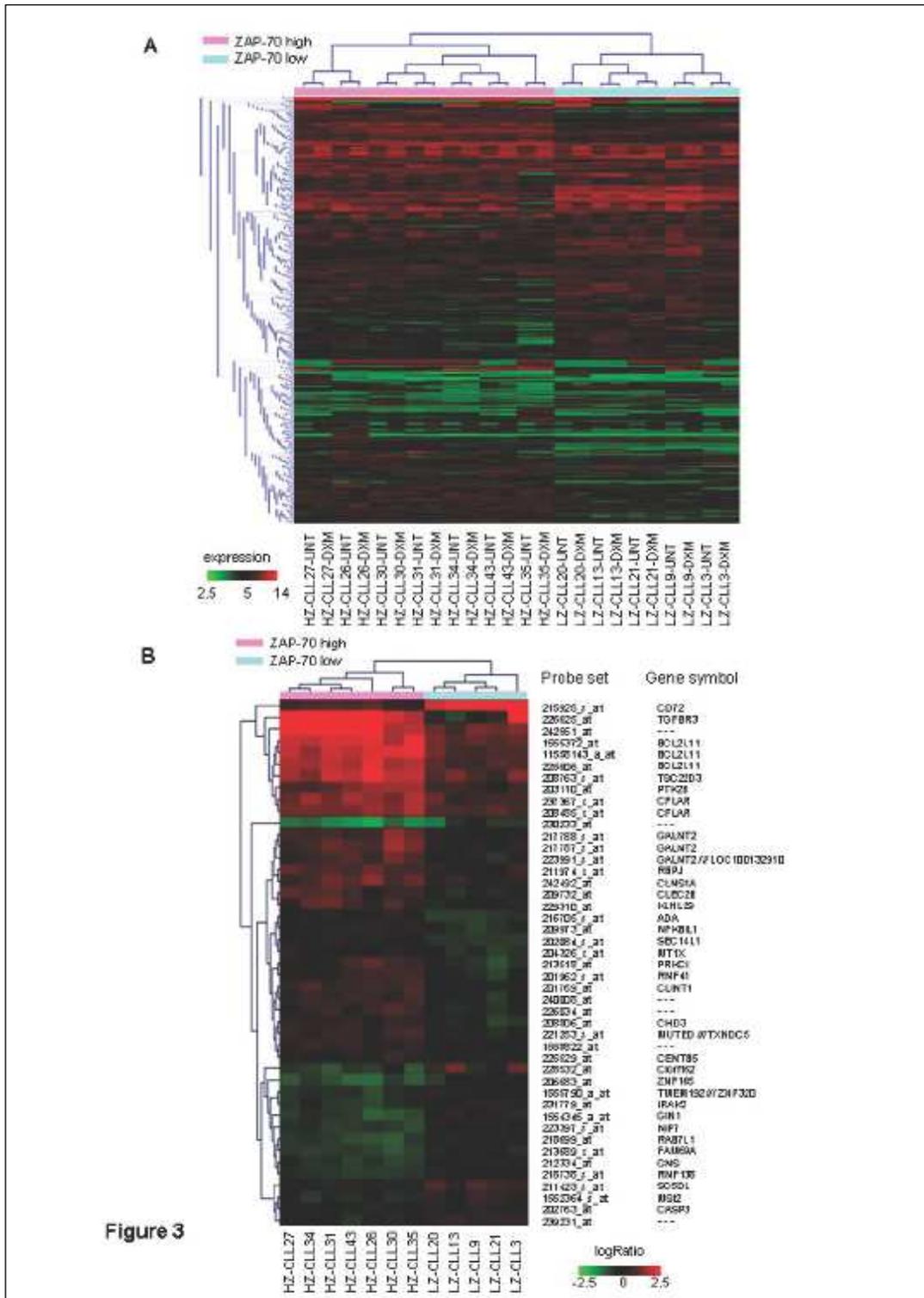
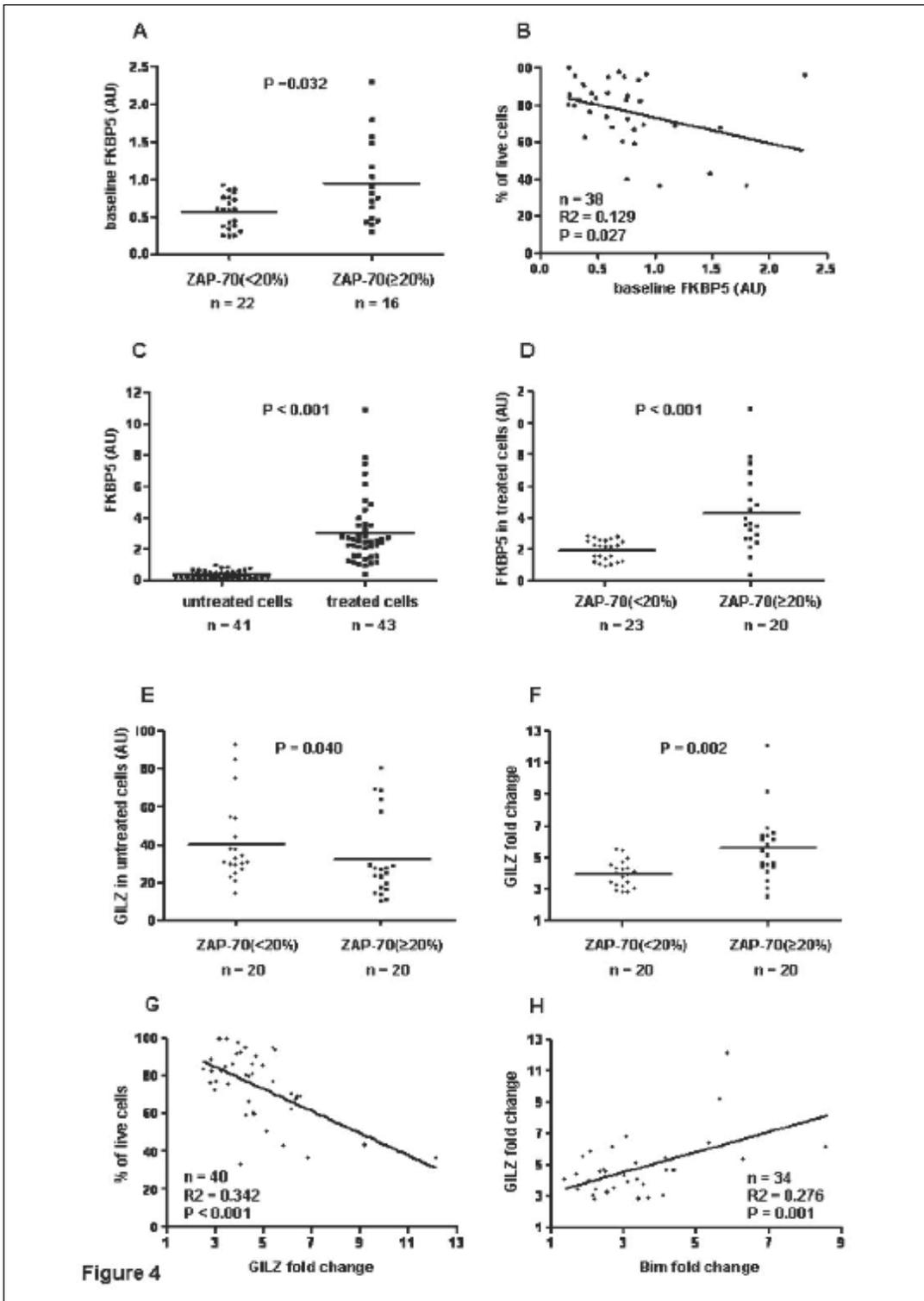


Figure 2





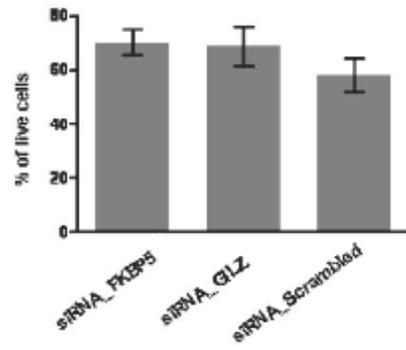


Figure 5

Supplementary table 1. Clinic-biological characteristics and response to the treatment with dexamethasone of the series of patients with CLL

Sample number	Gender	Age (years)	13q14.3 deletion	11q22.3 deletion	17p13.1 deletion	trisomy 12	ZAP70 (%)	IGHV category	Live cells (%)
1	M	71	yes	no	no	no	6	MCLL	94
2	F	71	no	no	no	no	7	MCLL	90
3*	F	44	yes	no	no	no	5	MCLL	100
4	M	80	no	no	no	yes	2	MCLL	93
5	M	70	no	no	no	no	10	MCLL	81
6	M	50	yes	no	no	no	5	MCLL	78
7	M	64	yes	no	no	no	1	MCLL	94
9	M	85	yes	no	no	no	4	MCLL	99
9*	M	71	no	no	no	no	2	MCLL	100
10	F	40	yes	no	no	no	11	MCLL	89
11	M	48	yes	no	no	no	0	MCLL	84
12	F	40	yes	no	no	no	6	MCLL	70
13*	F	47	yes	no	no	no	2	MCLL	99
14	F	50	no	no	no	no	8	MCLL	90
15	M	41	yes	no	no	no	15	MCLL	49
16	M	56	no	no	no	no	3	MCLL	78
17	M	69	no	no	no	no	0	MCLL	75
18	M	62	yes	no	no	no	4	MCLL	74
19	M	45	yes	no	no	no	2	MCLL	88
20*	M	63	no	no	no	no	3	MCLL	85
21*	M	68	yes	no	no	no	6	MCLL	91
22	M	56	no	no	no	no	1	MCLL	86
23	F	70	yes	no	no	no	12	MCLL	74
24	M	53	yes	no	no	no	10	MCLL	93
25	M	58	yes	yes	yes	no	77	UCLL	52
26*	M	30	no	no	no	no	73	UCLL	53
27*	F	60	no	no	no	no	35	UCLL	66
28	M	48	no	no	no	no	80	UCLL	83
29	M	57	no	yes	no	no	04	UCLL	70
30*	M	55	no	no	no	yes	60	UCLL	64
31*	M	54	yes	no	no	no	36	UCLL	52
32	M	72	no	no	no	no	30	UCLL	61
33	M	74	no	no	no	yes	90	UCLL	89
34*	M	61	yes	no	yes	no	51	UCLL	42
35*	M	61	yes	no	no	no	61	UCLL	66
36	M	49	no	no	no	no	39	UCLL	76
37	F	70	no	no	no	yes	73	UCLL	86
38	F	48	no	yes	no	no	30	UCLL	50
39	M	48	yes	no	no	no	66	UCLL	64
40	F	41	yes	no	no	no	70	UCLL	66
41	M	46	no	no	no	yes	73	UCLL	66
42	M	58	no	no	no	no	70	UCLL	97
43*	F	69	yes	no	no	no	75	UCLL	66
44	M	45	yes	no	no	no	46	UCLL	76
45	M	79	no	no	no	no	26	UCLL	67
46	M	82	yes	no	no	no	46	UCLL	63
47	M	56	yes	no	no	no	5	UCLL	70
46†	F	63	yes	no	yes	no	1		80
48	F	54	yes	no	yes	no	50		84
50	M	67	yes	no	yes	yes	30		66

M: male; F: female; * CLL cases selected for GEP analysis; † CLL case with 17p13.1 deletion, low ZAP-70 expression and poor response to dexamethasone.