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Dissertação no âmbito do mestrado em Investigação Biomédica orientada pela Professora Doutora Ana Bela Sarmento Antunes da Cruz Ribeiro e pela Doutora Raquel Fernanda da Silva Alves e apresentada à Faculdade de Medicina da Universidade de Coimbra

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"Evolution forged the entirety of sentient life
using only one tool: the mistake".

Robert Ford in *Westworld*

Agradecimentos

Este trabalho é o resultado de muito esforço e sacrifício. Durante este meu percurso académico surgiram alguns obstáculos, quer pessoais quer profissionais, os quais me fizeram abrandar o ritmo e, muitas das vezes, desviar-me dos meus principais objectivos. É verdade que em sempre consegui conciliar o meu trabalho em part-time com o desenvolvimento da tese, levando à acumulação e ao atraso no trabalho. Desta forma, percebi que um mestrado deve ser encarado como um trabalho a "full-time" e não em "part-time" como muitas das vezes eu o fazia. Recordo-me perfeitamente da professora Ana Bela me dizer que, acima de tudo, seria necessária disponibilidade mental e foco para concluir este objectivo. No entanto, nem sempre tive essa mesma disponibilidade, o que levou a que esta etapa se prolongasse por demasiado tempo.

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Por fim quero agradecer a quem me apoiou incondicionalmente e foi a minha grande motivação nos últimos tempos, à Mariana, por todo o amor e carinho. Foste uma

agradável surpresa que veio mudar o rumo da minha vida e que tornou tudo bastante mais fácil!

O meu sincero obrigado a todos!

Abstract

Acute lymphoblastic leukemia (ALL) is a heterogeneous haematological neoplasia characterized by progressive and abnormal accumulation of lymphoblasts in the bone marrow, blood and extramedullary sites. This malignancy affects, predominantly, children which represents about 80 % of ALL cases, and the peak age is between 2 and 5 years of age. Genetic alterations that occur in lymphoid progenitor cells cause impairment of the normal differentiation process leading to proliferation of immature cells. In fact, the hallmarks of ALL are chromosomal aberrations and genetic alterations, that define genetic distinct groups. The identification of these structural alterations has been important for establishment of correct diagnostic, risk determination and development of targeting therapies.

JAK-STAT is an essential signalling pathway involved in many cellular processes, including regulation of hematopoiesis, lymphoid differentiation, cell proliferation and survival. Dysregulation of JAK-STAT has been implicated in the development of hematological malignancies, including ALL. Several genetic alterations in key components of this signalling cascade, including *JAK2* and *STAT5* mutations as well as mutations in *CALR* gene, activate constitutively JAK-STAT signalling. Aberrant activation of JAK-STAT signalling compromise the regulation of normal cellular processes leading to uncontrolled proliferation and survival of leukemic cells. Therefore, JAK-STAT pathway is an attractive therapeutic target for development of new drug inhibitors to treat this leukemia.

The aims of this work were to characterize five *in vitro* ALL models for *CALR* expression and mutations and evaluate the therapeutic potential of two JAK-STAT inhibitors Ruxolitinib (RUX) and Pimozide (PIM). For this purpose, we used five distinct *in vitro* models of ALL: 697 and REH cells as B-ALL models, and CEM, JURKAT and MOLT4 cells as models of T-ALL. The *CALR* expression was evaluated by qRT-PCR and *CALR* mutations by Sanger sequencing. To evaluate the therapeutic potential of RUX and PIM on cell viability, all cell lines were treated in presence and absence of increasing concentrations of both inhibitors in different therapeutic schemes and analysed by resazurin assay. Cell death was evaluated by flow cytometry (FC), using the double

staining of annexin-V and 7-AAD, and by optic microscopy, using May-Grünwald-Giemsa staining. Finally, cell cycle was analysed by FC, using propidium iodide staining of DNA content.

In terms of *CALR* expression, ALL cell lines presented similar levels of expression with exception of JURKAT cell line, where high *CALR* expression was detected. Additionally, no *CALR* mutations on exon 9 were found in any of these cell lines. The effect of RUX in ALL models was time, cell line and scheme of drug administration dependent. This drug presented a cytotoxic effect mediated by apoptosis activation and no cytostatic effect was observed in the studied cell lines. On the other hand, PIM revealed an effect time, dose, cell line and scheme administrations dependent in the five ALL models. Mechanistically, apoptosis was the predominantly mechanism of cell death, proved by flow cytometry and morphological analysis. Additionally, in JURKAT cell line, this inhibitor also induced a cytostatic effect, with cell cycle arrest at G_0/G_1 phase. The combination of both JAK-STAT inhibitors, RUX and PIM, in our models did not presented an additive or synergistic effect.

In conclusion, our results suggest that JAK-STAT inhibitors, RUX and PIM, may constitute a potential therapeutic approach in acute lymphoblastic leukemia, being the efficacy related with different genetic backgrounds.

Keywords

- Acute lymphoblastic leukemia
- JAK-STAT pathway
- Calreticulin
- Ruxolitinib
- Pimozide

Resumo

A leukemia linfoblástica aguda (LLA) é uma neoplasia hematológica heterogénea caracterizada pela progressiva acumulação de linfoblastos essencialmente na medula óssea e sangue periférico. Esta doença afeta, principalmente, crianças em cerca de 80% dos casos, e o auge da doença ocorre entre os 2 e os 5 anos de idade. Modificações genéticas que ocorrem nas células progenitoras da linhagem linfoide, provocam alterações no normal processo de diferenciação dos linfócitos, levando a que ocorra a proliferação anormal de células imaturas. De facto, a LLA é caracterizada por alterações cromossómicas e genéticas que definem grupos geneticamente distintos. A identificação destas alterações estruturais é de extrema importância, de modo a que seja feito um correto diagnóstico da doença, com vista à determinação do nível de risco e ao desenvolvimento de terapias dirigidas a estas alterações.

A via de sinalização celular JAK-STAT está envolvida em diversos processos celulares que regulam não só a hematopoiese e a diferenciação linfoide, mas também a proliferação e a sobrevivência celular. Alterações que ocorrem nesta via e que provocam uma desregulação da mesma, estão envolvidas no desenvolvimento de diversas doenças hematológicas onde se inclui a LLA. A aberrante ativação da via é provocada por alterações genéticas em diversos componentes onde se incluem mutações nos genes que codificam as proteínas JAK2, STAT5 e calreticulina. Este comprometimento da via leva a que ocorra uma desregulação nos normais processos que regulam a proliferação e sobrevivência celular, levando a uma proliferação e sobrevivência descontroladas de células leucémicas. Deste modo, a via de sinalização JAK-STAT constitui um alvo terapêutico bastante atrativo para o desenvolvimento de novos fármacos neste tipo de leucemia.

Este trabalho teve como principais objetivos a caracterização dos níveis de expressão do gene que codifica a calreticulina (*CALR*), assim bem como a identificação de possíveis mutações do mesmo gene nos 5 modelos de LLA. Adicionalmente, procurou-se avaliar o potencial terapêutico de dois fármacos inibidores da via JAK-STAT, o ruxolitinib (RUX) e o pimozide (PIM). Para este fim, foram utilizados 5 modelos de LLA, as linhas celulares 697 e REH que correspondem a células do tipo B, e as linhas CEM,

JURKAT e MOLT4 que são células do tipo T. A avaliação dos níveis de expressão do *CALR* foi feita através de PCR quantitativo em tempo real, e a deteção de mutações por sequenciação de Sanger. Para avaliar o potencial terapêutico dos fármacos RUX e PIM na viabilidade celular, as linhas celulares utilizadas foram expostas a diversas concentrações destes inibidores em diferentes esquemas terapêuticos que incluíram a administração em toma única e diária, assim como a associação terapêutica entre os dois fármacos. Posteriormente, foram analisados os níveis de proliferação recorrendo ao ensaio da resazurina. A morte celular induzida pelos fármacos foi analisada através de citometria de fluxo, usando a dupla marcação da anexina-V e 7-AAD, e por microscopia óptica, através da análise morfológica com a solução de *May-Grünwald-Giemsa.* Por fim, o ciclo celular foi analisado por citometria de fluxo, utilizando o marcador de ADN, o iodeto de propídio.

Relativamente à expressão do gene CALR, as linhas celulares em estudo apresentaram níveis de expressão semelhantes, com a excepção da linha celular JURKAT onde foi detectada uma maior expressão deste gene. Adicionalmente, não foi detectada nenhuma mutação envolvendo o exão 9 do gene *CALR* nas linhas celulares testadas. O RUX demonstrou ter um efeito dependente do tempo de incubação, da linha celular e do esquema de administração. Este fármaco teve um efeito citotóxico nas linhas celulares em estudo, mediado pela activação da apoptose, porém não apresentou qualquer efeito citostático. Por outro lado, o PIM também demonstrou ter um efeito não só dependente da dose administrada e do tempo, mas também da linha celular e do esquema de administração nestes modelos de LLA. A apoptose foi o principal tipo de morte induzida pelo PIM nas linhas celulares, tendo sido confirmada através de citometria de fluxo e da análise dos aspetos morfológicos característicos da morte celular por apoptose, por microscopia ótica. Adicionalmente, o PIM teve um efeito citostático na linha celular JURKAT, induzindo um bloqueio do ciclo celular na fase G_0/G_1. Para finalizar, a combinação dos dois fármacos, RUX e PIM, não teve qualquer efeito sinérgico nas linhas celulares.

Concluindo, estes resultados sugerem que os inibidores da via JAK-STAT, RUX e PIM, podem constituir uma promissora abordagem terapêutica na leucemia linfoblástica aguda, onde a sua eficácia poderá estar associada às diferenças genéticas.

Palavras-chave

- Leucemia linfoblástica aguda
- Via de sinalização JAK-STAT
- Calreticulina
- Ruxolitinib
- Pimozide

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Abbreviations

6-MP 6-mercaptopurine

7-AAD 7-aminoactinomycin D

A Adenine

ABL1 v-abl Abelson murine leukemia viral oncogene homolog 1

ABL2 – v-abl Abelson murine leukemia viral oncogene homolog 2

AGM Aorta-gonad-mesonephros

ALL Acute lymphoblastic leukemia

ALLO-SCT Allogeneic stem cell transplantation

AML Acute myeloid leukemia

AML1 Acute myeloid leukemia 1

AMPK AMP-activated protein kinase

AYAs Adolescents/young adults

B-ALL B-cell acute lymphoblastic leukemia

BBB Blood-brain barrier

BCL2 BCL2 apoptosis regulator

BCL-xl B-cell lymphoma extra-large

BCR Breakpoint cluster region

BM Bone marrow

C Cytosine

CALGB 8811 Cancer and Leukemia Group B 8811 trial

CALR Calreticulin

CCD Coiled-coil domain

CD1A Cluster of differentiation 1A

CD3 Cluster of differentiation 3

CD4 Cluster of differentiation 4

CD47 Cluster of differentiation 47

CD8 Cluster of differentiation 8

CDKN2A Cyclin dependent kinase inhibitor 2A

CDKN2B Cyclin dependent kinase inhibitor 2B

cDNA Complementary DNA

CLP Common lymphoid progenitor

CML Chronic myelogenous leukemia

CMP Common myeloid progenitor

CNS Central nervous system

CR Complete remission

CREBBP CREB binding protein

CRFL2 Cytokine receptor like factor 2

CSCs Cancer stem cells

CT Cycle threshold

D Aspartate

DBD DNA binding domain

ddNTPs Dideoxynucleotide triphosphates

DNA Deoxyribonucleic acid

DNMT3A DNA (cytosine 5) methyltransferase 3 alpha

dNTPs Deoxynucleotide triphosphates

DS-ALL Down-syndrome ALL

DUX4 Double homeobox 4

E Glutamate

E2A Immunoglobulin enhancer binding factors E12/E47

ECM Extracellular matrix

EGFR Epidermal growth factor receptor

EPOR Erythropoietin Receptor

ER Endoplasmic reticulum

ERG V-ets avian erythroblastosis virus E26 oncogene homolog

ET Essential thrombocythemia

ETP-ALL Early T-cell precursor ALL

ETV6 Ets variant 6

EZH2 Enhancer of Zeste 2 homolog 2

FAK Focal adhesion kinase

FBS Fetal bovine serum

FBXW7 F-box and WD repeat domain containing 7

FC Flow cytometry

FDA Food and drug administration

FLT3 Fms Related Receptor Tyrosine Kinase 3

G Guanine

GLOBOCAN Global cancer observatory

GMPs Granulocyte/monocyte progenitors

GPCR G protein-coupled receptor

GRB2 Growth factor-bond protein 2

GUSB Glucuronidase Beta

HCVAD Hyper-CVAD

HIV Human immunodeficiency virus

HSCs Hematopoietic stem cells

iAMP21 Intrachromosomal amplification of chromosome 21

IC_{50} Half maximal inhibitory concentration

IKZF3 IKAROS Family Zinc Finger 3

IL2RB Interleukin 2 Receptor Subunit Beta

IL-7 Interleukin 7

IL7R Interleukin 7 Receptor

IRS 1/2 Insulin receptor substrate 1/2

JAK Janus kinase

JH JAK homology domain

K Lysine

KMT2A Lysine (K)-specific methyltransferase 2A

L Leucine

LMO2 LIM domain only 2

LSCs Leukemic stem cells

LT-HSC Long-term hematopoietic stem cells

MCL1 Myeloid cell leukemia sequence 1

MDR Minimal residual disease

MDR1 Multi-drug resistance protein 1

MDS Myelo-dysplastic syndromes

MEF2D Myocyte-specific enhancer factor 2D

MEPs Megakaryoitic/erythoid progenitors

MLL Mixed lineage leukemia

MPL Thrombopoietin receptor

MPNs Myeloproliferative neoplasms

MPPs Multipotent progenitors

mTORC1 Mammalian target of rapamycin complex 1

NF1 Neurofibromatosis type 1

NGS Next-generation sequencing

NK Natural killer cells

OS Overall survival

P2RY8 Purinergic receptor P2Y, g-protein coupled 8

PAG1 Phosphoprotein Membrane Anchor 1

PAX5 Paired box 5

PBX1 pre-B-cell leukemia homeobox 1

Ph Philadelphia chromosome

PHF6 PHD Finger 6

Ph-like ALL Philadelphia chromosome like ALL

PI3K hosphatidylinositol-4,5-Bisphosphate 3-Kinase

PIAS Protein inhibitors of activated STATs

PIM Pimozide

PMF Primary myelofibrosis

PS Phosphatidylserine

PTPs Protein tyrosine phosphatases

PV Polycythemia vera

qRT-PCR Quantitative real-time polymerase chain reaction

RB1 RB Transcriptional Corepressor 1

RNA Ribonucleic acid

RPMI-1640 Rowell Park Memorial Institute 1640 medium

RTK Receptor of tyrosine kinase

RUNX1 Runt-related transcription factor 1

RUX Ruxolitinib

SEM Standard error of the mean

SH2B3 SH2B Adaptor Protein 3

SHC SH2-domain containing protein

SIRPα Signal regulatory protein alpha

SOCs Suppressors of cytokine signalling

SOS Son of sevenless

STAMs Signal-transducing adapter molecules

STAT Signal transducer and activator of transcription

ST-HSC Short-term hematopoietic stem cell

STIP STAT-interacting protein

T Tyrosine

TAL1 T-cell acute leukemia 1

T-ALL T-Cell Acute lymphoblastic leukemia

TCF3 Transcription factor 3

TEL Translocation Ets Leukemia

TF Transcription factor

TKIs Tyrosine kinase inhibitors

TLX1 T-cell leukemia homeobox 1

TLX3 T-cell leukemia homeobox 3

TP53 Tumor Protein P53

TSLP Thymic stromal lymphopoietin

TYK2 Tyrosine Kinase 2

WBC White blood cell

WHO World health organization

ZNF384 Zinc finger protein 384

Chapter 1 - Introduction

1.1. Cancer

Cancer is the most lethal disease in the world and is expected an increase in the incidence, as consequence of population growth and aging. As a multifactorial disease, it is known that lifestyle misbehaviours such as poor diet, absence of physical activity and smoke habits are risk-increasing factors to cancer development. In 2015, the World Health Organization (WHO) classified cancer as leading cause of death for people younger than 70 years-old in 48 of 172 countries assessed, including most of the European countries as Portugal (1). Moreover, and according with Global Cancer Statistics (GLOBOCAN), in 2018 was expected 18.1 million cancer new cases and 9.6 million of deaths globally (1). While lung is the top cancer, in incidence and mortality globally, leukemia represents 2.4% of new cases (437 033 new cases) and 3.2% of estimate deaths (309 006 deaths) per year in both genders, respectively (**Figure 1**). The 5-year prevalence of leukemia overcomes one million cases in total worldwide (1).

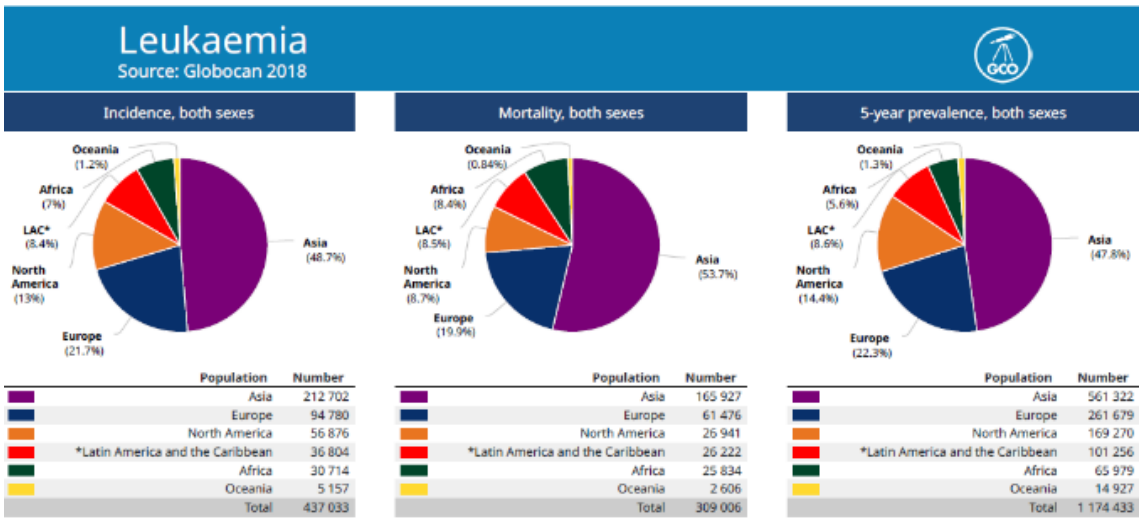

Figure 1 – Worldwide leukemia incidence, mortality and 5-year prevalence in 2018. While Asia is the top continent with higher number of incidence, mortality and 5-year prevalence, Europe accounts second higher number in all these three indicators (Adapted from GLOBOCAN 2018 – Leukemia (1)).

The population of Portugal is not growing as the world does but is becoming old as well. As consequence of Portugal population´s aging, cancer incidence and mortality is increasing very quickly over the years. Statistics from GLOBOCAN revealed that in 2018, the number of new cases was 58 199 and the number of cancer deaths was 28 960. The most frequent cancers in Portugal, ranked by cases, are prostate cancer in males, breast in females and colorectum in both sexes. The number of leukemia new

cases is not high as prostate, breast or colorectum cancers, but represents 2% of new cases, and 3.3% of the patients died from this malignancy in that year (1). Taking all these data and statistics into consideration, it is urgent to develop new and more efficient approaches to fight cancer.

Cancer is a heterogeneous condition and every single cell of the human body has the potential to exhibit cancer properties in certain conditions. In fact, a large diversity of cancers diverges in their behaviour and response to therapies due to this heterogeneity (2–4). Genetic and epigenetic events, such as genetic mutations and epigenetic modifications, are responsible for cancer heterogeneity and influence its progression and therapeutic outcomes. Single-base sequence changes, gene fusions or chromosomal rearrangements are examples of lesions that contribute to genetic instability (5,6). Moreover, the interaction between cancer cells and microenvironment is also an important concept in cancer heterogeneity since cancer cells phenotype and function depends on their niche. Niches with favourable characteristics select and promote growth of cancer cells (2–4).

Different evolutionary processes of cancer have been suggested. The clonal evolution theory argues that cancer development is an evolutionary process, in which mutated cells have selective advantages comparatively with non-mutated cells (2,7). A cascade of genetic alterations produces a dominant clone and cells evolving from this clone have the same oncogenesis potential. While driver mutations are crucial for cancer initiation, cooperating mutations also contribute to subsequent clonal expansion. However, these cells can show different mutational and phenotypic features within subclones, resulting in cancer heterogeneity (**Figure 2**) (2,8).

Epigenetics is also important process for cell development and a source of functional heterogeneity within cells. Mechanisms that promote functional changes in the genome without altering DNA sequence, such as DNA methylation or histone modification, modulate gene expression are essential to stem cell state maintenance (3). Self-renewal is a crucial feature for maintenance and growth of stem cell population, where cells divide through asymmetric or symmetric division producing one or two daughter cells, respectively. Molecular programs that control self-renewal and proliferation create developmental hierarchies and impose cell fate specification in normal tissues (3). In cancer, stem cell maintenance pathways are dysregulated, and

self-renewal capacity is enhanced. Cells with high replicative potential and extensive self-renewal are called cancer stem cells (CSCs). In the cancer stem cell model, CSCs are a population of cells responsible for sustaining clonal expansion and can induce functional heterogeneity through differentiation (**Figure 2**)(2,3).

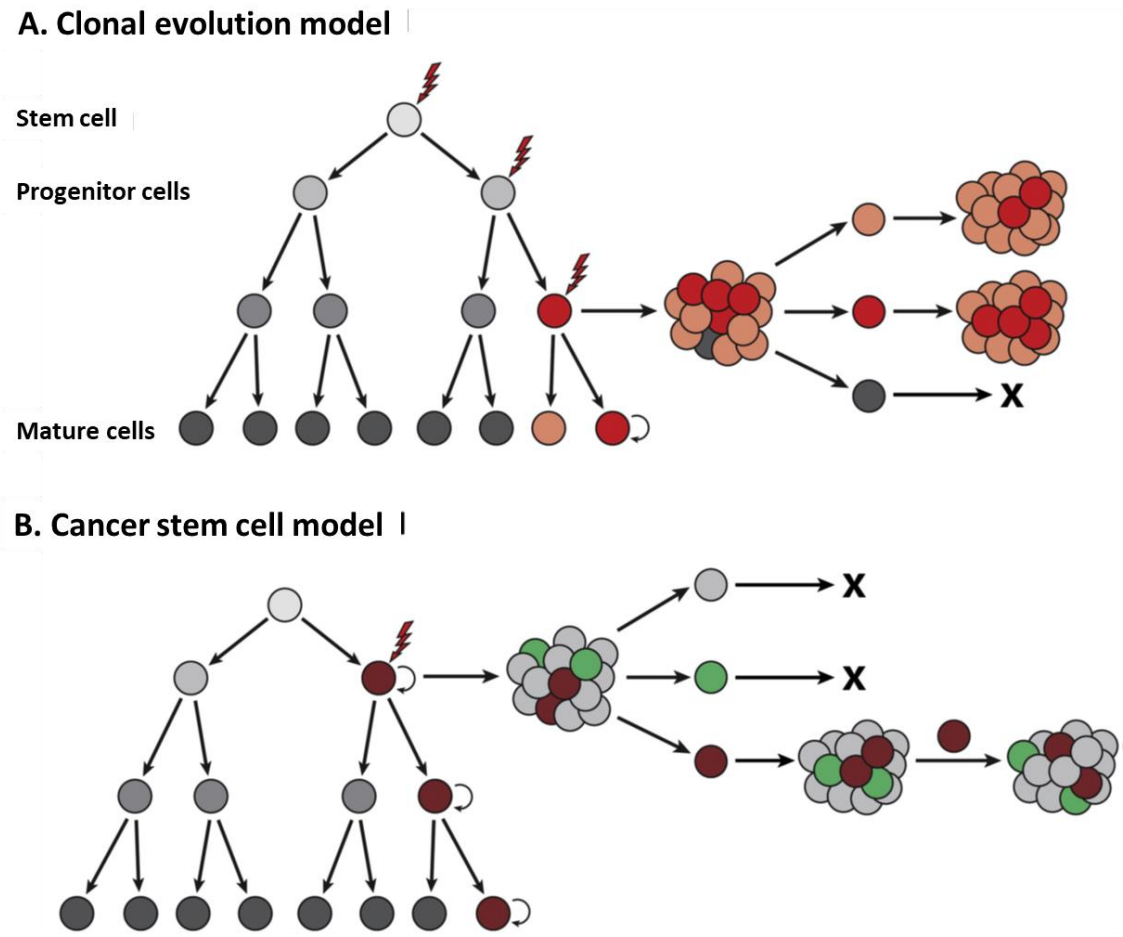

Figure 2 - Clonal evolution and cancer stem cell models. A. In the clonal evolution model, a sequence of mutations produces a dominant clone (red cell) with selective growth advantages comparatively with non-mutated cells (grey cells). This dominant clone produces different subclones (red and orange cells) with the same oncogenesis potential. **B**. In the cancer stem cell model, cancer stem cells (brown cells) are responsible for sustaining clonal expansion and create functional heterogeneity (brown and green cells) through differentiation (Adapted from Visvader *et. al.,* 2012 (2).

CSCs are responsible for therapy resistance, failure and risk of relapse due to their genetic and epigenetic heterogeneity (2,3,8). Conventional anti-cancer therapies, such as chemotherapy or radiotherapy are, in some cases, ineffective against CSCs. Some of these cells are in a quiescent state, a state where cells are in dormancy and do not divide, and antiproliferative drugs do not affect them. Consequently, quiescent CSCs can survive

to anticancer therapy and, depending on the stimuli, can re-enter in cell cycle and proliferate. This event can be an opportunity to quiescent cells survive and grow since competing high proliferative cells are eradicated by treatment. On this purpose, some therapies may act as a selective pressure for surviving cells, creating resistance against anticancer treatments and promoting proliferation (3,8).

1.2. Hematopoiesis

Blood is responsible for many functions that goes from the transport of oxygen to the mediation of the immune response, containing a variety of cells that are responsible to perform this duty. Since all blood cells have a restricted life-span, they must be constitutively produced during lifetime in an event called hematopoiesis (9,10). Hematopoiesis is a hierarchical process where all blood cells are produced from hematopoietic stem cells (HSCs) present in the bone marrow (BM) (11–13). HSCs hold special properties that allows them to produce more HSCs (self-renewal) and to differentiate into blood progenitor cells (12). These cells face several differentiation and maturation processes, culminating in mature cells such as erythrocytes, monocytes, granulocytes, lymphocytes and megakaryocytes (10,11,13).

The location where hematopoiesis occurs changes throughout human development and, for this reason, it can be divided into embryonic and adult hematopoiesis (**Figure 3**). During embryonic development, hematopoiesis is initiated in the yolk sac and red blood cells are originated from hemangioblast. This process later occurs in the aorta-gonad-mesonephros (AGM) region and placenta. Here, hemogenic endothelial cells originate HSCs with self-renewal capacity and the first blood cells progenitors. Afterwards, fetal liver momentarily hosts hematopoiesis before it reaches

Figure 3 - Different localizations of hematopoietic development. Embryonic hematopoiesis is initiated in the yolk sac and is reallocated to placenta and aorta-gonad-mesonephros (AGM) region. The adult hematopoiesis occurs in the bone marrow (Adapted from Rieger *et. al* 2012 (9)).

its final destination, the BM. This niche is from now on responsible for the regulation of adult hematopoiesis and HSCs produced, with different self-renewal and differentiation properties. For instance, while adult HSCs are in a quiescent state, fetal HSCs are cycling in order to produce blood cells faster, crucial to embryonic development (9–11).

Hematopoiesis is a strictly regulated process. HSCs fate is controlled not only by the niche and external signals, such as cytokines or other signalling molecules, but also by internal molecules including transcription factors (TFs) (9,11,13). TFs and epigenetic modifications promote changes in gene expression that may induce HSCs self-renewal, migration, proliferation, differentiation or apoptosis (**Figure 4**) (13–15). Differentiation of HSCs into mature blood cells involves specific factors that determinate lineage commitment and restriction, inhibiting opposite factors. This repression is important to maintain lineage differentiation stable without interposition of another molecular pathways (9–11,13).

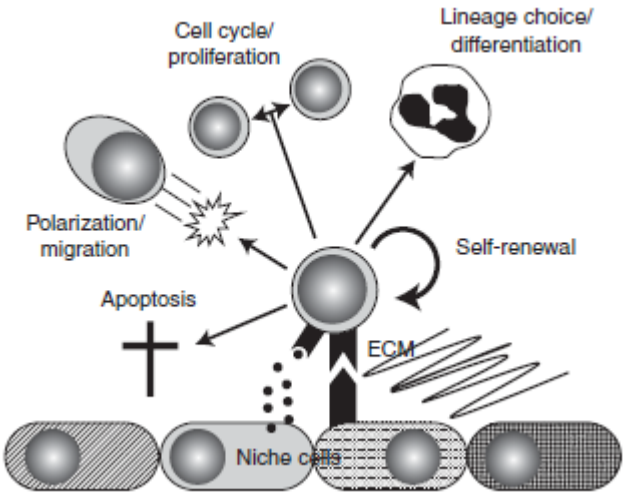

Figure 4 - Regulation of HSCs fate by niche and external signal. Cell-fate decisions are influenced by different external factors, including cytokines and the extracellular matrix (ECM). External factors promote gene expression modifications that may induce HSCs to apoptosis, polarization/migration, proliferation, differentiation or self-renewal (Adapted from Rieger *et. al* 2012 (9)).

The microenvironment of BM is composed by diverse cell types with specific functions that regulate HSCs fate and control blood progenitors production (9,16,17). The cells that constitute BM niche are responsible to transmit signals to HSCs and included endothelial cells, mesenchymal cells, osteoblasts, macrophages and nerve cell (16,17). Factors secreted by these cells directly or indirectly influence maintenance and expansion of HSCs (16,17).

In adult hematopoiesis, HSCs are in a state of quiescence which is important to keep the integrity of their functions and to avoid errors derived from cell cycle (9,11,17). However, quiescent HSCs can re-enter in cell cycle and proliferate responding to the microenvironment signals. In this manner, the signals and instructions provided by BM niche are essential to regulate HSC differentiation/proliferation to the demands of the blood system.

The myeloid and lymphoid lineages are the two main branches in this hierarchy of the hematopoietic tree (**Figure 5**) (18,19).

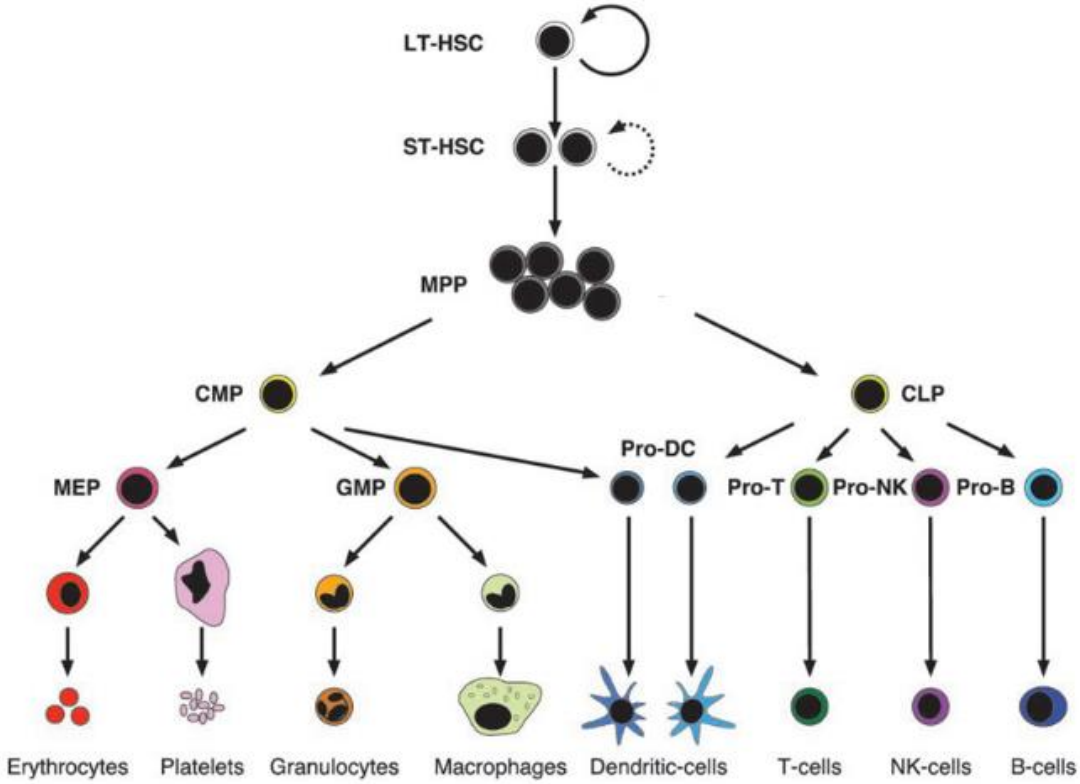

Figure 5 – Differentiation and lineage commitment in hematopoiesis. Long-Term HSCs (LT-HSCs) are highly self-renewing cells. These cells can branch into short-Term HSCs (ST-HSCs) that have limited self-renewal capacity. ST-HSCs give rise to multipotent progenitors (MPPs), which are cells that can generate all mature blood cell types. However, these cells can only support haematopoiesis transiently because they do not have self-renewal capacity. MPPs can differentiate in common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). CMP is the progenitor of myeloid, megakaryocyte and erythroid lineages. Therefore, it induce the production of megakaryiotic/erythroid progenitors (MEPs), which give rise to erythrocytes and platelets, and granulocyte/monocytes progenitors (GMPs), which differentiate into granulocytes and monocytes. The CLP has the capacity to generate T lymphocytes (T-cells), B lymphocytes (B-cells) and natural killer cells (NK-cells) (Adapted from Passegué *et al*. 2003 (18)).

HSCs are divided into long-Term (LT-HSCs), which are highly self-renewing cells, and these derivate into short-Term (ST-HSCs) that have limited self-renewal capacity.

Moreover, ST-HSCs differentiate into multipotent progenitors (MPPs) that are unable of self-renewing, but capable to differentiate into more restricted progenitors, as the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). The CMP is the progenitor of myelo-erythroid lineages. It induces the production of megakaryiotic/erythroid progenitors (MEPs), which give rise to erythrocytes and platelets, and granulocyte/monocytes progenitors (GMPs), which differentiate into granulocytes and monocytes. The CLP is able to generate T lymphocytes (T-cells), B lymphocytes (B-cells) and natural killer (NK) cells. Both myeloid and lymphoid branches can give rise to dendritic cells.

A well-balanced hematopoiesis is crucial to supply blood with the right number of mature cells needed and to avoid leukemogenesis. While regulation of HSCs self-renewal, differentiation and programmed cell death (apoptosis) is important to maintain the right number of proliferating and differentiating cells, dysregulation of these molecular programs may result in malignancy. HSCs and blood progenitors can be transformed into CSCs, or more particularly leukemic stem cells (LSCs), due to cytogenetic abnormalities, epigenetic changes and accumulation of genetic mutations (18,20,21). In virtue of these alterations, LSCs manifest stem cell properties, such as limitless self-renewal, proliferative and differentiation capacities that allows them to survive (21,22). Moreover, epigenetic changes such as changes in DNA methylation and histone modifications may contribute to leukemogenesis since they regulate gene transcription. Cancer cells are hypomethylated globally and hypermethylated in gene promoters regions, which may result in silencing of tumor suppressor genes (20). LSCs can be derived from HSCs and also from a committed progenitor cell (**Figure 6**) (21,22).

HSCs that harbour genetic and/or epigenetic abnormalities contribute to transformation of HSCs into LSCs that maintain an undifferentiated state (**Figure 6A**). On the other hand, alterations can occur in a common progenitor cell which reacquire stem cell properties (**Figure 6B**).

LSCs possess mechanisms that are responsible for its resistance to conventional therapies, contributing for therapeutic failure. The expression of proteins such as multi-drug resistance protein (MDR1) helps LSCs to eliminate cytotoxic drugs within the cell (22,23). Moreover, LSCs express CD47, which is a cell surface protein that interacts with

the signal regulatory protein alpha (SIRPα) present in macrophages, promoting the inhibition of phagocytosis (22,24).

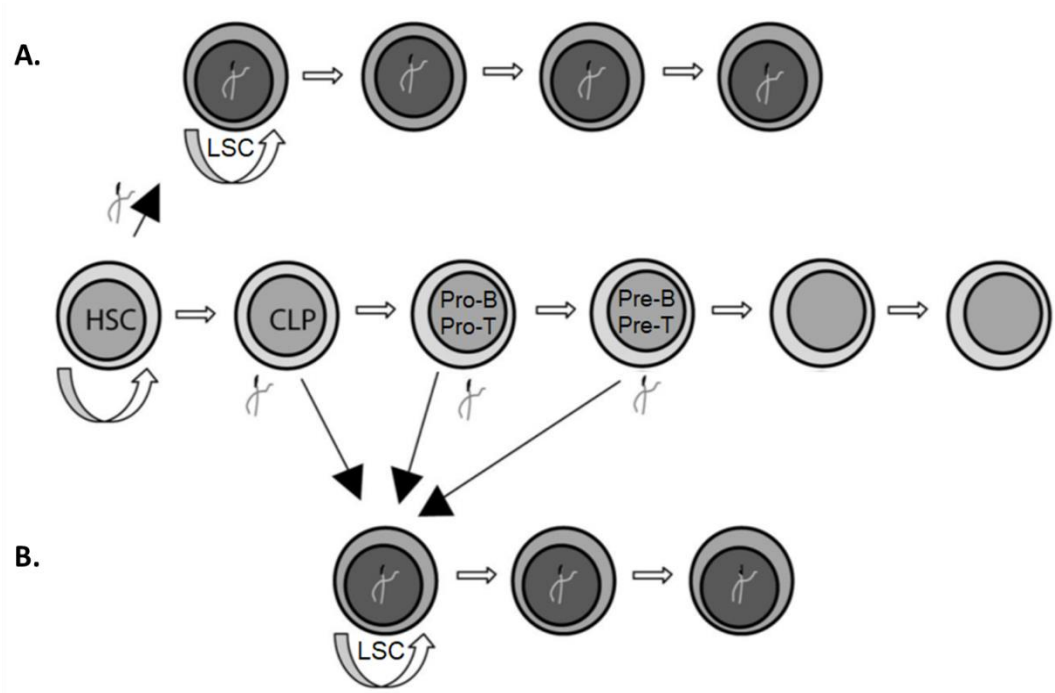

Figure 6 – Leukemia initiating cell. A. Dysregulation of genetic mechanisms lead to leukemic transformation (LSC) and maintenance of an undifferentiated state. B. Genetic alterations occur in lymphoid progenitors (CLP, pro- and pre-B or -T) which gain stem cell properties such as self-renewal and uncontrolled proliferation. (Adapted from Bernt *et. al* 2009 (21)).

The BM microenvironment is critical for support and regulation of HSCs. Similarly, the BM microenvironment is responsible for maintenance and development activity of LSCs and their descendent clones (17). Yet, LSC are not completely dependent on niche signals to proliferate and survive, as HSCs are. Moreover, these leukemic cells can reprogram the BM niche to support their development, and eliminate normal HSCs (17). On these cases, allogeneic stem cell transplantation alongside with high doses of chemotherapy and radiotherapy is used eliminate invading LSCs and restore normal hematopoiesis.

Classification and characterization of hematological malignancies is crucial to have an efficient diagnosis, prognosis and an adequate therapy. Moreover, genetic characterization and molecular diagnostic tools are also essential in this categorization. In general, hematological neoplasias are divided into myeloid neoplasms, such as myeloproliferative neoplasms (MPNs), myelodysplastic syndromes (MDS), acute

myeloid leukemia (AML); and lymphoid neoplasms, such as mature T and B-cell neoplasms, NK-cell neoplasms, Acute lymphoblastic leukemia (ALL), and Hodgkin lymphoma (25,26). ALL is divided into B-cell ALL and T-cell ALL, according with cells affected, being categorized in B-cell lymphoblastic/lymphoma not otherwise specified and with recurrent genetic abnormalities and early T-cell precursor lymphoblastic leukemia (25,27).

1.3. Acute lymphoblastic leukemia

1.3.1. Epidemiology and predispose factors

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease characterized by a progressive and abnormal accumulation of lymphoid progenitor cells in the BM, blood and extramedullary sites (28). As a result of an impaired maturation caused by genetic alterations that occur in the lymphoid progenitors, the normal differentiation process is arrested leading to an uncontrolled proliferation of immature cells (28,29). This disease occurs mainly in children, representing about 80% of the cases, and the peak age is between 2 to 5 years old, and after the age of 50 (28–31). The prevalence of childhood ALL is higher in males than females and the survival rates these of patients when treated with standard protocols are 90% and around 40% in adults (29,30).

The exact mechanisms beyond the development of ALL are unknown. However, it is believed that leukemogenesis results from interactions between endogenous and environmental factors (29,30,32). While some cases, around 5 % are directly associated with inherited genetic syndromes including Down´s syndrome, the majority apparently have no identified hereditary causes (29,30,33). On the other hand, extrinsic factors such as exposure to ionising radiation, chemicals (e.g. pesticides) and certain viruses such as Epstein-Barr Virus or Human Immunodeficiency Virus (HIV), also contributes to the development of ALL (28,30,32,34,35). Curiously, the events succeeding the atomic bombs in Japan are a true proof that exposure to ionising radiation is a serious factor for ALL progress (28–30).

Besides endogenous and environmental factors, there are predisposing factors that are also important to mention. Gender, age, white cell count and immunophenotype are classical risk factors, however parental habits such as tobacco or alcohol use, diet and high birthweight of new-born are important factors to take into account (32,36).

1.3.2. Pathophysiology

ALL is a malignant disease characterized by the abnormal proliferation of undifferentiated cells in BM, blood and extramedullary sites (28,29,32). The malignant transformation of hematopoietic lymphoid progenitors is caused by impairments in maturation and differentiation processes which leads to abnormal proliferation and accumulation of leukemic cells (29).

Advances in molecular techniques allowed a better understanding of the ALL pathogenesis. Consequently, ALL is promoted not only by endogenous and exogenous factors, but also by genetic alterations and cytogenetic modifications. These alterations occur typically in genes that are fundamental for cellular homeostasis and regulation. Genes involved in important processes such as the self-renewal, differentiation and apoptosis contribute for leukemic-like changes of hematopoietic stem cells and blood cell progenitors (26,28–30).

The hallmark of ALL are chromosomal aberrations and genetic alterations (28,37). Despite the fact of aberrant chromosomal alterations, such as translocations and rearrangements, are thought to be the initiating events in ALL pathogenesis, these modifications are not enough to induce leukemia. In fact, there are several additional cooperating events such as DNA copy number alterations and genetic mutations, that are required for the development of this disease (**Figure 7**) (29,30,38).

In 2016, the WHO published the new classification of acute leukemia based on information provided by scientific and clinical studies from multiple countries (25). The aim of this classification is to define ALL subtypes with clinical significance based on clinical information, morphology, immunophenotyping, cytogenetics and genetics (25,27). Immunophenotyping of malignant cells present in the BM and peripheral blood is important to distinguish and define cell lineage (32). ALL include multiple subtypes and this technique provides information that is fundamental for the establishment of

correct diagnosis, risk classification and development of target therapies (37). Therefore, ALL can be divided into two main branches accordantly with developmental markers present in cell surface: B-cell precursor acute lymphoblastic leukemia (B-ALL) and T-cell precursor acute lymphoblastic leukemia (T-ALL) (25).

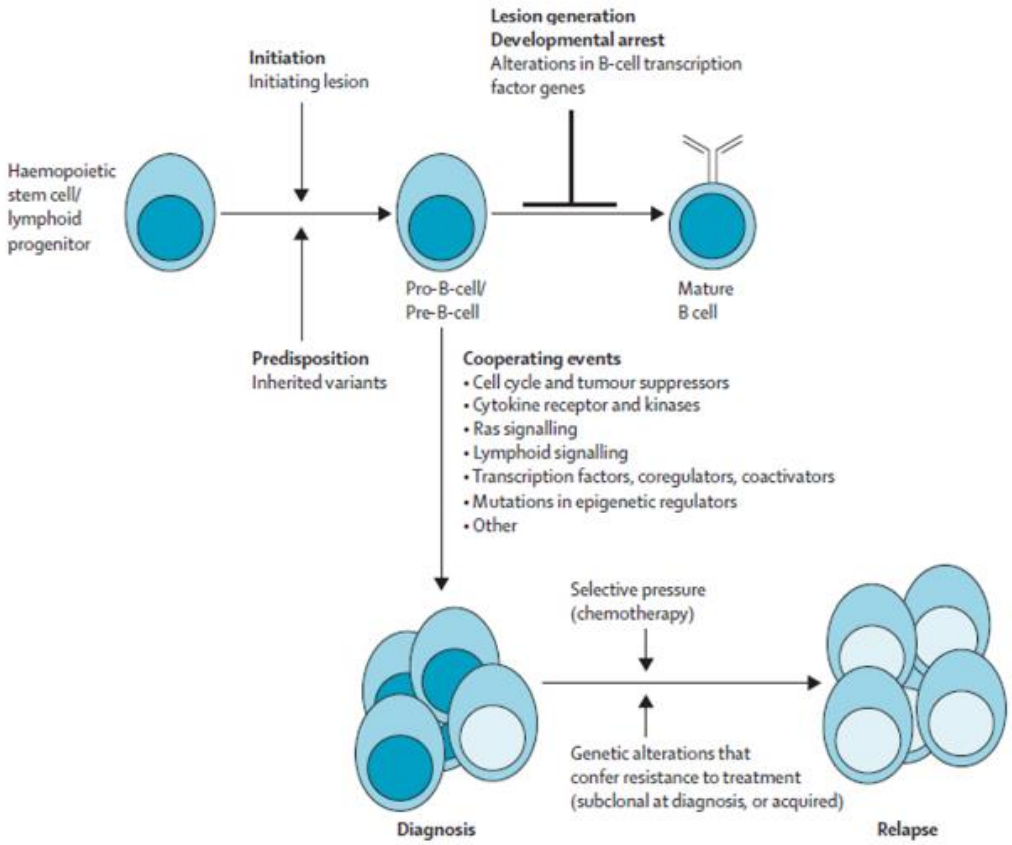

Figure 7 - Development and evolution of ALL, taken B-cell ALL as example. Chromosomal abnormalities alongside with predisposition factor are initiating lesions that contribute to leukemic transformation of lymphoid progenitors. These alterations are responsible for impairment of the maturation and differentiation processes which leads to developmental arrest and an abnormal proliferation of Pro-B and Pre-B-cells. Consequently, leukemic cells accumulate in the BM leading to a suppression of normal hematopoiesis and infiltration of extramedullary sites. Cooperating events contribute to additional malignancy leukemic cells. Genetic mutations in genes involved in cell cycle and tumour suppressor genes promote dysregulation of cell proliferation and differentiation. Moreover, additionally genetic alterations in key factors, such as cytokine receptors and transcription factors, involved in lymphoid development pathways contribute to perturbation of them. Leukemic cells are polyclonal and genetically distinct at diagnosis. In fact, treatments can be a selective pressure for the emergence of subclones. Initial therapy can suppress and eliminate more dominant and proliferative clones, however subclones can acquire genetic alterations that confer resistance to treatment. Therefore, subclones that become dominant are responsible for disease relapse (Adapted from Inaba *et al.* 2013 (29)).

B-ALL is the most common type of ALL, both in children and adults, and is defined by the malignant transformation of lymphoid B-cell progenitors (39). Clinical improvements have been made over the years, and most of the patients achieve good outcomes with a combination of conventional therapies. These outcomes are greater in children than adults. While >95% of young patients enter in complete remission (CR), between 60% and 85% of adults achieve the same goal (30,39). Regarding the overall survival (OS), children achieve values above than 80% and adults values below than 50% (39,40).

B-ALL is characterized by the presence of cytogenetic alterations marked by chromosomal abnormalities such as aneuploidy, chromosomal rearrangements that originate fusion genes, and intrachromosomal amplifications (**Table 1**) (37,39). These alterations are present in about 75% of B-ALL cases and are important for diagnosis, risk stratification and treatment planning (37,41). Some of these genetic alterations, such as high-hyperdiploidy (between 51 and 65 chromosomes) and the translocation between chromosomes 12 and 21 (that encodes for the gene *ETV6-RUNX1*) which are present in 25-30% of childhood cases, are associated with good prognosis (37,39). Moreover, the t(1;19)(q23;p13) translocation (that encodes the fusion gene *TCF3-PBX1/E2A-PBX1*) which is present in 5% to 6% of children and adults with B-ALL cases, and is also associated with good prognosis (42). On the other hand, hypodiploidy, t(9;22)(q34;q11.2) translocation (that originates Philadelphia (Ph) chromosome), Philadelphia chromosome-like, and *KMT2A/MLL* translocations are associated with worse outcomes and risk of relapse (43–45).

Hypodiploidy (<44 chromosomes) is associated with a poor outcome, and can be subdivided into three groups: high hypodiploidy (42-44 chromosomes), low hypodiploidy (31 to 39 chromosomes) and near haploidy (24 to 30 chromosomes) (46). Patients with high hypodiploidy have better prognosis than those with low hypodiploidy and near haploidy (46). Moreover, the mutation profile of low-hypodiploid and near-haploid ALL are different (45). In 71% of the cases, patients that display low-hypodiploidy exhibit additional somatic mutations in genes involved in receptor tyrosine kinase and RAS signalling (such as *NF1*, histone modifiers, *CREBBP*, *CDKN2A/B*, *IKZF3* and *PAG1*) (45). In low hypodiploidy, mutations in *TP53* gene occurs in about 91% of

childhood and adult cases, among others genes such as *IKZF2*, *RB1*, histone modifiers and *CDKN2A/B* (45).

Table 1 - Frequency and clinical significance of cytogenetic abnormalities in B-ALL.

ALL subtype	Genetic alterations	Frequency and clinical significance	[Reference]
B-ALL	**Hyperdiploidy (>50 chromosomes)**	25%-30% in clindren and 7-8% in adults Favourable outcome	[37]
	Hypodiploidy (<44 chromosomes)	2%-3% of patients; 6% in children and 7-8% in adults. Poor prognosis	[45,46]
	t(12;21)(p13;q22) [*ETV6-RUNX1/ TEL-AML1*]	25-30% in children and up to 4% in adults Favourable outcome in children	[37,39]
	t(1;19)(q23;p13) [*TCF3-PBX1/ E2A-PBX1*]	1-6% in children and 1-3% in adults Favourable outcome	[42]
	t(9;22)(q34;q11.2) [*BCR-ABL1*]	1-5% in children and 25-50% in adults Poor prognosis improved with use of TKIs	[37,43]
	Ph-like ALL	15% of childhood and 20-25% of adult cases Poor prognosis	[25,47-49]
	***CRLF2* rearrangement [IGC-CRLF2/P2RY8-CRLF2]**	8% of pediatric cases Common in Down syndrome-associated Poor prognosis	[50-52]
	***KMT2A* (*MLL*) rearrangements**	More frequente in infants, 1-2% in older children, and 4-9% in adults Poor prognosis	[44,53]
	***DUX4*- and *ERG*-deregulated ALL**	7% of B-ALL cases and 5% of pediatric cases Good prognosis	[54]
	***MEF2D*-rearranged ALL**	3-4% of childhood and 6% of adult cases Poor prognosis	[55]
	***ZNF384*-rearranged ALL**	7% of pediatric and 15% of adult cases Intermediate prognosis	[56,57]
	***PAX5* rearrangements**	32% of B-ALL cases No clinical outcomes	[53,58]
	iAMP21 (intrachromosomal amplification of chromosome 21)	More common in older children Poor prognosis	[59-61]

B-ALL – B-cell acute lymphoblastic leukemia; ***ETV6*** – Ets variant 6; ***RUNX1*** – Runt-related transcription factor 1; ***TEL*** – Translocation Ets Leukemia; ***AML1*** – Acute myeloid leukemia 1; ***TCF3*** – Transcription factor 3; ***PBX1*** – pre-B-cell leukemia homeobox 1; ***E2A*** – Immunoglobulin enhancer binding factors E12/E47; ***BCR*** - Breakpoint cluster region; ***ABL1*** – v-abl Abelson murine leukemia viral oncogene homolog 1; **Ph-like ALL** – Philadelphia chromosome like acute lymphoblastic leukemia; ***CRFL2*** – Cytokine receptor like factor 2; ***P2RY8*** – Purinergic receptor P2Y, g-protein coupled 8; ***KMT2A*** – Lysine (K)-specific methyltransferase 2A; ***MLL*** – Mixed-lineage leukemia; ***DUX4*** – Double homeobox 4; ***ERG*** – v-ets avian erythroblastosis virus E26 oncogene homolog; ***MEF2D*** – Myocyte-specific enhancer factor 2D; ***ZNF384*** – Zinc finger protein 384; ***PAX5*** – Paired box 5; **iAMP21** – Intrachromosomal amplification of chromosome 21.

The incidence of *BCR-ABL1* translocation in children is 5% and increases with age reaching about 50% of older patients cases (37,43). This chromosomal abnormality creates a fusion protein that is a non-receptor tyrosine kinase. Ph-chromosome was associated with poor prognosis when treatment was based on conventional chemotherapy. However, the outcomes increases with the introduction of tyrosine kinase inhibitors (TKIs) combined with conventional therapeutic schemes (37).

In 2016, the WHO recognized a new B-ALL subtype also associated with worse outcomes and risk of relapse, called *BCR-ABL1*-like ALL, or also referred to as Philadelphia chromosome-like (Ph-like) ALL (25). This subtype is characterized by the absence of *BCR-ABL1* rearrangement, but patients express the same genetic profile as those who display *BCR-ABL1* effectively. Ph-like represent 15-20% of ALL cases and is associated with a low rates of OS (47). Moreover, the incident of Ph-like varies accordantly with age, affecting 15% of children and 20-25% of adolescents and adults (48).

A study from Roberts et al. identified that Ph-like ALL comprise multiple genetic alterations that affect cytokine receptor genes and tyrosine kinases (49). These activating alterations of kinases are present in 91% of patients with Ph-like ALL and frequencies varied with age. The most common alteration is the *CRLF2* rearrangement present in 49.7% of the patients, followed by genetic alterations involving JAK-STAT signalling pathway (*IL7R, FLT3, SH2B3, JAK1, JAK3, TYK2* and *IL2RB*), and fusions involving *ABL1* and *ABL2* genes, both present in 12.6% of the cases. *JAK2* rearrangements are frequent in young adults representing 7.4% of the cases. This study reported also mutations involving RAS pathway and associated with hypodiploidy in 4.3% of cases, as well as rearrangements of *EPOR* in 3.9%. Regarding the fact that kinase-activating lesions are frequent in Ph-like patients, they can beneficiate from TKIs based therapies as Ph-chromosome positive patients (49).

CRLF2 rearrangements are common in Ph-like and Down-syndrome associated B-ALL, affecting about 8% of pediatric patients and more than 50% of the patients with Down-syndrome (50). They occur usually with the immunoglobulin heavy chain locus creating *IGH-CRLF2*, and less common deletions creating *P2RY8-CRLF2* fusion genes. These rearrangements culminate in overexpression of CRLF2 protein (51). Additionally, CRLF2 overexpression is correlated with constitutive activation of several signalling pathways such as JAK-STAT, PI3K/mTOR and BCL2 (52). Although *CRLF2* alterations are associated with high risk B-ALL and poor prognosis, treatments targeting abnormal activated pathways have been efficient.

KMT2A/MLL rearrangements are most common in infants (less than a year of age) and are related with poor outcome. Moreover, the incidence in children and adults varies between 1-2 % and 4-9 %, respectively (53). Alterations in the histone methylation

of *KMT2A/MLL* genes promote the leukemic transformation of HSCs and lymphoid progenitors (44).

Deregulation of *DUX4* (double homeobox 4 gene) and *ERG* (ETS transcription gene) comprises about 7% of B-ALL cases and is associated good prognosis (54). *DUX4* rearrangement leads to the aberrant expression of DUX4 that binds to *ERG* gene. These rearrangements and deletions of *ERG* gene, present in about 5% of pediatric cases, promote the expression of an aberrant protein that is a competitive inhibitor of the wild-type *ERG,* resulting in changes of ERG transcriptional activity (54).

Rearrangements of myocyte enhancer factor 2D (*MEF2D*) gene is a B-ALL subtype present in 3-4% and 6% of children and adult cases, respectively (55). *MEF2D* is rearranged with multiple genes which results in increase of *MEF2D* transcriptional activity and therapeutic resistance due to deregulation of *MEF2D* target genes. This alteration is associated with poor outcome.

The zinc finger 384 (*ZNF384*) rearrangement accounts 7% of pediatric and 15% of adult cases and has an intermediate prognosis (56,57). This alteration creates a gene fusion involving transcriptional regulators and chromatin modifiers. Moreover, *ZNF384* rearrangement upregulates the JAK-STAT pathway and patients with this alteration can benefit from TKIs.

Alterations in genes involved in B-cell development such as *PAX5* are found in about 32% of B-ALL and do not have clinical impact (53). Rearrangements with *ETV6* and *JAK2* (2.5% of childhood cases), and other alterations such as deletions and inactivating mutations result in loss of function of *PAX5* (58).

The intrachromosomal amplification of chromosome 21 (iAMP21) is associated with poor prognosis and low OS (59). iAMP21 is common among older children and adolescents, but uncommon in pediatric (2%) and adult B-ALL patients (60). Although iAMP21 can be associated with poor prognosis, treatment intensification improve the outcomes (61).

Besides primary cytogenetic abnormalities, B-ALL comprises additional genetic alterations involving different intermediates of signalling pathways. The most common alterations are found in transcription factors involved in lymphoid development such as *PAX5* and *IKZF1*, which are present in 40% of B-ALL cases (62). Mutations and rearrangements of *PAX5* with other genes such as *ETV6* and *JAK2* are driver alterations

to development of B-ALL (58,62). Mutations in *IKZF1* gene are frequent in B-ALL cases, occurring in 80% and 70% of Ph and Ph-like cases, respectively. *IKZF1* deletions promotes the expression of a negative form of Ikaros protein that inhibits the wild-type Ikaros. The loss of function of the wild-type Ikaros results in the arrest of lymphoid differentiation, contributing for B-ALL development (63).

B-ALL include further genetic alterations in tumor suppressor genes, cell cycle regulators, epigenetic factors and in genes involved in developmental pathways (such as cytokine receptors genes, kinase genes and transcriptional regulation genes) which contribute to leukemic transformation of B-progenitor cells.

T-ALL is a malignancy characterized by accumulation of immature cells in the BM, which are derived from early T-cell progenitors and express immature T cell markers (64). Approximately 15% of T-ALL cases occur in children, while 25% occur in adults (30,64). Even though T-ALL used to be considered a high-risk group, intensification of therapeutic protocols improved the outcomes to 80% in children and 60% in adults (32,65). However, the prognosis is inferior for patients that have a poor response to the initial treatment (32). Similar to B-ALL, T-ALL results from genetic alterations that promote the malignant transformation of T-cell progenitors into leukemic cells (64). These alterations result in developmental arrest and dysregulation of important pathways that control cell growth and survival, proliferation, and differentiation.

T-ALL is divided in three subgroups based on genetic alterations and thymocyte immunophenotype which varies at different stages of development (**Figure 8**) (66). Early T-cell precursor (ETP) ALL is a subgroup characterized by immature differentiation of thymocytes and reduced expression of T-cell markers such as CD4 and CD8 (67). Moreover, ETP cells exhibit abnormal expression of HSCs and myeloid markers (67). ETP ALL affects about 10 % of children and between 40% and 50% of adults T-ALL cases, being associated with poor prognosis (67,68). The second group exhibit a different immunophenotype than ETP-ALL, being CD1a, CD4 and CD8-positive cells (69). These immunophenotype markers are associated with good prognosis and are expressed in early stages of cortical thymocyte maturation (69). The last group are CD3, CD4 and CD8 positive cells, reflecting a more mature stage (late cortical) of thymocyte development (**Figure 8**) (66).

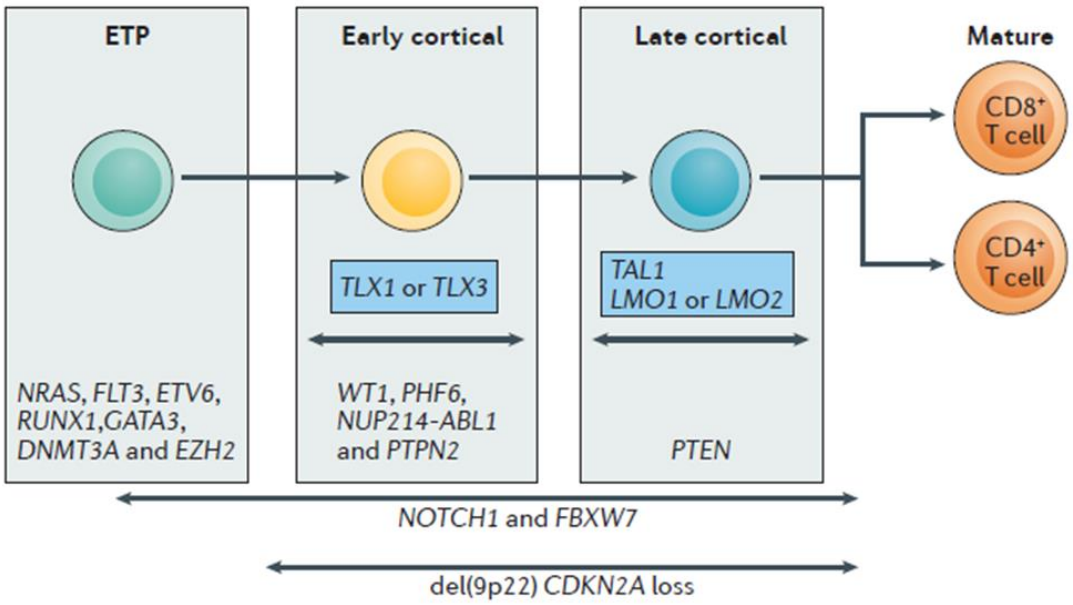

Figure 8 – Different stages of thymocyte development and recurrent genetic alterations distinguish T-ALL groups. Early T-cell precursors (ETPs) are characterized by reduced expression of CD4 and CD8 T-cell markers and abnormal expression of myeloid and hematopoietic markers. Genetic alterations in ETP group (green) include activating mutations of signalling factors such as *NRAS* and *FLT3,* and inactivating mutations of transcription factors such as *ETV6*, *RUNX1* and *GATA3*, and epigenetic regulators such as *DNMT3A* and *EZH2.* Moreover, this group have low incidence of alterations in NOTCH1 signalling (*NOTCH1* and *FBXW7*) and cell cycle regulators, including *CDKN2A.* Thymocytes arrested in early cortical stage (yellow) which are CD1a, CD4 and CD8-positive, display genetic alterations that activate oncogenes such as *TLX1* and *TLX3.* This group show high incidence of NOTCH1 signalling associated alterations and del(9p22) resulting in *CDKN2A* loss. Additional alterations include mutations in *WT1*, *PHF6* and *PTPN2* genes, and *NUP214-ABL1* rearrangement. Thymocytes arrested in late cortical stage of development (blue) and exhibit a CD3, CD4 and CD8-positive immunophenotype, express aberrantly *TAL1*, *LMO1* and *LMO2* genes. Moreover, this group of T-ALL exhibit frequently mutations in *NOTCH1*, *FBXW7* and *PTEN* genes. (Adapted from Belver *et al.* 2016 (64))

Genetic abnormalities including chromosomal rearrangements, mutations that activate or inactivate transcription factors (TFs), and deletions are alterations that dysregulate and contribute for pathogenesis of T-ALL (**Table 2**) (70). Gene expression profiling allowed the identification of genetic alterations in T-ALL. These include rearrangements and mutations that aberrantly activate TFs such as *TAL1*, *LMO2, TLX1*, *TLX3*; mutations that inactivate cell cycle regulators such as *CDKN2A* and *CDKN2B*; alterations that promote dysregulation of NOTCH1 signalling (*NOTCH1* and *FBXW7* genes); and dysregulation of JAK-STAT pathway (activating mutations of *IL7R*, *JAK1*, *JAK3* and *STAT5*) (64,70).

Table 2 - Frequency and clinical significance of genetic alterations in T-ALL.

ALL subtypes	Genetic alteration	Frequency and clinical significance	[Reference]
T-ALL	**NOTCH1 signalling pathway:** • *NOTCH1* rearrangements and activating mutations • *FBXW7* inactivating mutations	• 50% of pediatric and 57% of adult cases • 14% of both pediatric and adult cases • Favourable outcome	[71-75]
	Cell cycle: • *CDKN2A* 9p21 deletion • *CDKN2B* 9p21 deletion	• 61% of pediatric and 55% of adult cases • 58% of pediatric and 46% of adult cases • Favourable outcome	[70,71,74]
	Transcription factors: • *TAL1* rearrangements and mutations • *LMO2* rearrangements and deletions • *TLX1* rearrangements and deletions • *TLX3* rearrangements	• 30% of pediatric and 34% of adult cases • 13% of pediatric and 21% of adult cases • 8% of pediatric and 20% of adult cases • 19% of pediatric and 9% of adult cases • *TAL1*, *TLX1* alterations are associated with good outcome, while *TLX3* with poor outcome; *LMO2* has no significative clinical impact	[64,66,74,76-78]
	JAK-STAT signalling pathways: • *IL7R* activating mutations • *JAK1* activating mutations • *JAK3* activating mutations • *STAT5B* activating mutations	• 10% of pediatric and 12% of adult cases • 5% of pediatric and 7% of adult cases • 8% of pediatric and 12 of adult cases • 6% of both pediatric and adult cases • Poor outcome	[79-87]
	Epigenetic factors: • *PHF6* inactivating mutations/deletions • *EZH2* inactivating mutations/deletions • *DNMT3A* inactivating mutations	• 19% of pediatric and 30% of adult cases • 12% of both pediatric and adult cases • 1% of pediatric and 14% of adult cases • Poor outcome	[70,80,88-92]

T-ALL – T-cell acute lymphoblastic leukemia; ***FBXW7*** – F-box and WD repeat domain containing 7; ***CDKN2A*** – Cyclin dependent kinase inhibitor 2A; ***CDKN2B*** – Cyclin dependent kinase inhibitor 2B; ***TAL1*** – T-cell acute leukemia 1; ***LMO2*** – LIM domain only 2; ***TLX1*** – T-cell leukemia homeobox 1; ***TLX3*** – T-cell leukemia homeobox 3; ***IL7R*** – Interleukin 7 receptor; ***JAK1*** – Janus kinase 1; ***JAK3*** – Janus kinase 3; ***STAT5B*** – Signal transducer and activator of transcription 5B; ***PHF6*** – PHD Finger 6; ***EZH2*** – Enhancer of Zeste 2 homolog 2; ***DNMT3A*** – DNA (cytosine 5) methyltransferase 3 alpha.

The most common alterations in T-ALL are abnormalities affecting NOTCH1 signalling pathway, which are present in more than 50% of the cases (71). NOTCH1 is a transmembrane protein that allows the transduction of extracellular signals to the nucleus which promotes transcriptional changes in target genes (72). Activation of NOTCH1 receptor is fundamental for T-cell fate specification and thymocyte development. Despite the fact that t(7;9)(q34;q34.3) translocation is responsible for expression of a truncated form of NOTCH1 which activates aberrantly NOTCH1 signalling, mutations in *NOTCH1* gene induce the same effect (71,73). Additionally, mutations of *FBXW7* gene inhibits the degradation of NOTCH1 active form, occurs in about 15% of T-ALL cases, and contributes for abnormal activation of the pathway (71). Moreover, cooperative alterations that occur in suppressor genes such as *CDKN2A* and *CDKN2B* increase the leukemogenicity of abnormal activated NOTCH1 signalling (74).

Genetic abnormalities targeting NOTCH1 signalling pathway are associated with good outcomes (75).

About 60% of T-ALL cases are characterized by 9p21 deletions leading to the inactivation of *CDKN2A* and *CDKN2B* genes and to the consequently loss of cell cycle control and T-ALL transformation (70).

In T-ALL, chromosomal abnormalities can lead to the ectopic expression of specific TFs including *TAL1*, *LMO2*, *TLX1* and *TLX3*, which are the most common TFs involved in oncogenic activity (66,76). Chromosomal alterations such as t(1;14)(p32;q11) lead to the abnormal expression of *TAL1* which is an important regulator gene of HSCs development (66,74). Expression of *TAL1* in T-ALL cases contribute for developmental arrest of thymocytes and consequently accumulation of immature cells (66). Moreover, chromosomal rearrangements that promote aberrant expression of *LMO* genes (*LMO1* and *LMO2*) in T-ALL are associated with late stages of T-ALL development and have a cooperative oncogenic role when co-expressed with *TAL1* (66).

The t(10;14)(q24;q11) is present in 5% to 10% and in 30% of children and adults T-ALL cases, respectively (66,77). This chromosomal translocation lead to high expression of *TLX1* in T-ALL, culminating in thymocyte arrest at late cortical stage (77). On the other hand, t(5;14)(q35;q32) translocation, which is present in about 25% of pediatric and 5% of adult T-ALL cases, induce high expression of *TLX3* (64,66,77,78). *TLX1* expression is associated with good prognosis and low risk of relapse, while aberrant expression of *TLX3* is associated with bad prognosis and high prevalence of relapse (66,77,78).

Multiple signalling pathways are involved in regulation of thymocyte development, including PI3K-AKT-mTOR, RAS-MAPK and JAK-STAT signalling pathways (64,79). These pathways control key cellular processes such as regulation of cell growth, proliferation, and survival of T-cell progenitor cells.

Genetic alterations in key components of JAK-STAT pathway including *JAK1*, *JAK2*, *JAK3* tyrosine kinases, *STAT5* transcription factor and *IL7R* receptor genes, lead to the abnormal activation of this pathway (80,81). JAK-STAT activation by IL-7 induces phosphorylation of JAK1 and JAK3, leading to the activation of STAT5, and increases cell proliferation and survival (82). Mutations in *IL7R* are found in about 10% of T-ALL, being more common in ETP T-ALL comparatively with late stages of thymocyte development, and leading to activation of STAT5 (83,84). Downstream of IL7R receptor, activating

mutations in *JAK1* and *JAK3,* as well as in *STAT5,* are described in about 10% of T-ALL cases (82,85,86). Additionally, the t(9;12)(p24;p13) translocation which encodes for *ETV6-JAK2* fusion gene produces a constitutively active kinase protein leading to activation of JAK-STAT (87).

Constitutive activating of JAK-STAT pathway results in dysregulation of proliferation and survival of leukemic cells, being associated with poor outcomes (79).

Epigenetic alterations with higher incidence in T-ALL involve *PHF6*, *EZH2* and *DNMT3A* epigenetic factors. *PHF6* gene is involved in cell cycle regulation (88). Inactivation of *PHF6* in T-ALL promotes leukemic transformation as a result of loss of PHF6 which is a tumor suppressor protein (88,89). *EZH2* gene encodes for EZH2 enzyme which is an epigenetic repressor involved in regulation of histones (90,91). Loss of function mutations in this gene are found in 25% of T-ALL cases and contribute for leukemic transformation of thymocytes through abnormal activation of NOTCH1 signalling (91). Additionally, these alterations are associated with poor prognosis (91). *DNMT3A* is epigenetic factor responsible for DNA methylation modifying (70,80). Mutations in DNMT3A gene are described in about 5 % of T-ALL, particularly in ETP-ALL, being associated with poor prognosis (92).

Thus, ALL (B-ALL and T-ALL) is a malignancy that comprise multiple subtypes which are defined by structural alterations that establish genetically distinct groups. The identification of structural abnormalities and determination of high-risk groups are important for diagnosis, risk determination and development of target therapies.

1.3.3. Diagnosis

Diagnosis of ALL is made through analysis of morphological and immunophenotypic aspects of lymphoblasts (36). Accumulation of undifferentiated lymphoblasts in BM, peripheral blood and extramedullary sites are features of ALL. In fact, diagnosis is defined by the occurrence of 20% or more undifferentiated lymphoblasts in BM or peripheral blood (28,93). Clinical symptoms include anemia, thrombocytopenia, fever, weight loss, fatigue, dyspenia and infections (28,32,93).

1.3.4. Prognostic factors

The main prognostic factors with clinical value include age, gender (in children), white blood cell count, immunophenotype, cytogenetics and genetics, and response to treatment (**Figure 9**) (28,94,95).

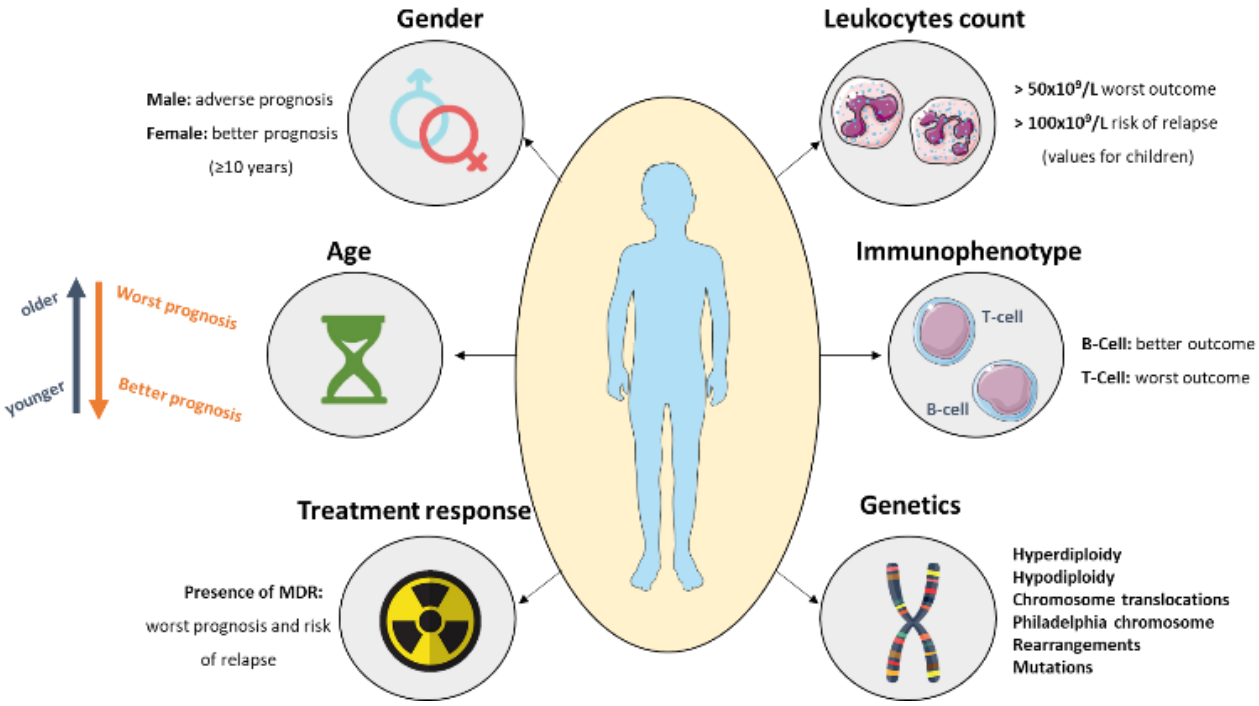

Figure 9 – Multiple prognostic factors contribute to risk stratification. Ages ≥10 years old and high leukocyte count are biological factors predictive of a worst prognosis. In children, males have worst outcomes than females. The immunophenotype can be predictive of outcome, being B-ALL associated with better outcome than T-ALL. Genetic alterations and minimal residual disease (MDR) are the most important prognostic factors. While hyperdiploidy and *ETV6-RUNX1* are associated with good prognosis, Philadelphia chromosome and Ph-like are associated to poor outcomes. Moreover, the presence of MDR is usually associated with worse outcomes and risk of relapse.

Usually, aging and high leukocyte count are predictive of poor outcomes. Children aged before 10 years-old have better prognostic than infants, adolescents and adults. People with age above 60 years-old have the worse outcomes due to treatment intolerance and comorbidities (30,32,94). Regarding leukocyte count, values above 50 x 10^9/L are unfavourable for children, and values higher than 100 x 10^9/L are linked to risk of relapse (30,32). Moreover, gender has been associated to different outcomes in children, where males present poorer outcomes than females (30,96). However, these results are not translated to adults.

The immunophenotype is also a valuable prognostic factor. B-cell ALL represents 75% of adult cases and is associated with a favourable outcome, comparatively with T-cell immunophenotype that is associated with worse outcomes (28,30,94).

Cytogenetic alterations are important not only for prognosis and risk determination, but also for development of targeted therapeutic strategies (28,94).

Evaluation of minimal residual disease (MDR) is important for risk stratification and to establishment of sequential treatments (97). MDR is used to detect and quantifying residual leukemic cells present in BM and peripheral blood. The methods used for MDR detection include cytogenetics, flow cytometry and polymerase chain reaction, being the later sensitive enough to detect one leukemic cell *per* 10,000 normal cells (98). Leukemic cells can be distinguished from normal cells through detection of immunoglobulin gene rearrangements which are common in B-ALL, and T-cell receptor gene rearrangements, common in T-ALL (98,99). Moreover, common gene fusions (*BCR-ABL1*, for instance) and immunophenotypes are useful for the same purpose (98,100). Nowadays, MDR is one of the most important prognostic factor in ALL. In fact, MDR evaluation at different time points is an important prognostic factor in pediatric and adult ALL (100,101). The most important time points to measure MDR are during and at the end of induction therapy, and at the beginning of consolidation therapy. Thus, MDR negativity (absence of leukemic cells) during induction therapy is associated with favourable outcome and can be a result of high sensitivity to the therapy (97). Moreover, MRD evaluation at early consolidation phase is crucial to decide if allogeneic transplant is appropriate for the patient due transplant associated mortality and toxicity (102,103).

1.3.5. Therapeutic Approaches

The treatment of ALL consist in three different phases: induction of remission, intensification (or consolidation), and continuation (or maintenance) (**Figure 10**)(28,29,32). The primary purpose of induction phase is to eliminate more than 99 % of initial leukemic cells and restore normal hematopoiesis (28,29,32). This phase has a duration between 4 and 6 weeks and can provide a remission in 96-99% of children and 78-93 % of adults (104,105). In fact, therapeutic schemes used in adults are adapted from pediatric regimens (106). Usually, children with intermediate-risk disease are administrated with at least three drugs regimen composed by glucocorticoids

(prednisone or dexamethasone), vincristine, and asparaginase or anthracycline (doxorubicin or daunorubicin) (29,32,107). High-risk children and most of adult cases are submitted to therapies with four or more drugs (106,107).

In the Cancer and Leukemia Group B 8811 trial (CALGB 8811), the induction phase has a duration of 4 weeks. During this period, it is administrated at least five drugs which are composed by cyclophosphamide on the first day, followed by three consecutive days of daunorubicin, vincristine and L-asparaginase (once and twice a week, respectively), and three consecutive weeks of prednisone (108). About 85% of the patients who received this treatment achieved complete remission with an overall survival of 69 % at 3 years (108).

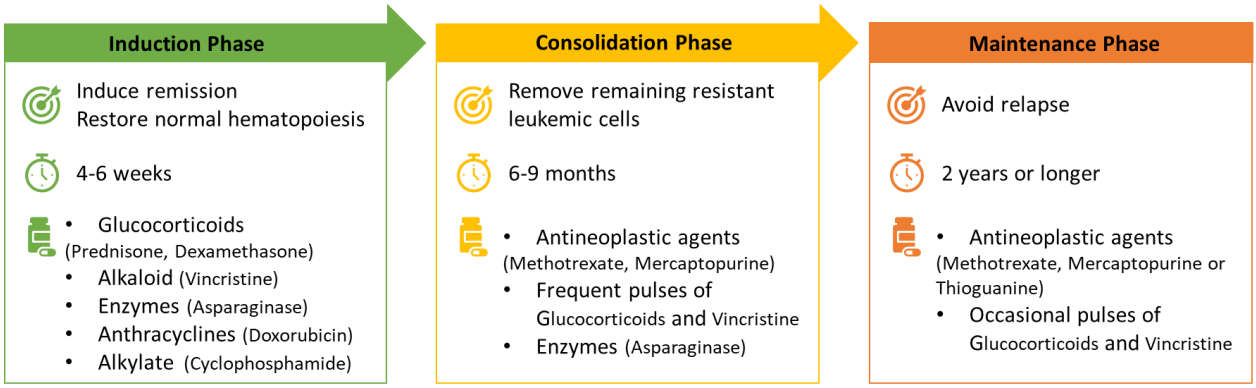

Figure 10 – ALL therapeutic schemes. ALL treatment is composed by three phases: induction, consolidation, and maintenance. The purpose of induction phase, which is 4 to 6 weeks longer, is to achieve complete remission and restore normal hematopoiesis. For this purpose, cyclophosphamide, vincristine, doxorubicin, dexamethasone and asparaginase are frequently used on this phase. The consolidation phase has a duration between 6 and 9 months and is used to remove the remaining resistant leukemic cells, reducing the risk of relapse. High doses of methotrexate and mercaptopurine are frequently used as antineoplastic agents, alongside with frequent pulses of glucocorticoids and vincristine, and high dose of asparaginase. The maintenance phase is composed by daily administration of mercaptopurine or thioguanine and weekly methotrexate, with occasional (if needed) pulses of vincristine and dexamethasone. This phase lasts 2 years but can be longer and is used to prevent relapse. Data derived from the following references 28,29,32,105-110,115.

In MRC UKALL XII/ECOG 2993 trial, the induction phase is divided into 2 stages of 4 weeks each (109). In stage 1, it is administrated a single dose of intrathecal methotrexate as central nervous system (CNS) prophylaxis since leukemic cells usually infiltrate into CNS being a source of relapse. The second stage is composed by cyclophosphamide, cytarabine, 6-mercaptopurine (6-MP) and 4 intrathecal doses of methotrexate. Moreover, patients receive radiotherapy if leukemic cells were detected

in CNS. The intensification of therapy followed the induction phase and it was composed by 3 cycles of methotrexate and L-asparaginase. After this stage, the patients who achieved complete remission were attended to consolidation and maintenance phase. However, patients with high-risk ALL were submitted to allogeneic stem cell transplantation (allo-SCT). In this trial, the complete response rate was 91% and overall 5-year survival was 38% (109).

The Hyper-CVAD (HCVAD) induction phase comprise 4 cycles of hyperfractinated cyclophosphamide, vincristine, doxorubicin and dexamethasone, alternated with 4 high dose cycles of cytarabine and methotrexate (110). For CNS prophylaxis is administrated 4 to 16 doses of intrathecal chemotherapy. The complete remission response on this trial was 92% and 32% overall 5-year survival. Similar with ECOG 2993, after induction phase, patients may go to intensification/consolidation followed by maintenance therapies or go to allo-SCT.

The presence of the *BCR-ABL1* is associated with worse outcomes and treatment with TKIs, such as imatinib, dasatinib and ponatinib, are beneficial in this period (111–113). The introduction of TKIs during induction phase in combination with traditional chemotherapy, such as HCVAD, lead to 90% of complete remission in *BCR-ABL1* positive patients (112,114). The combination of HCVAD with imatinib improved 3-year overall survival from 15% to 54%, comparatively with HCVAD alone (111). Moreover, incorporation of dasatinib, a second generation TKI, in HCVAD chemotherapy, improved complete remission rates to 96% and 5-year overall survival to 46% in patients who acquired imatinib resistance (112). Additionally, ponatinib, a third generation TKI, achieved better 3-year overall survival and event free survival, comparatively with dasatinib (113).

Intensification or consolidation phase follows induction once remission is achieved and normal hematopoiesis is re-established (29,32). The main goal of this phase is to remove the remaining leukemic cells that are resistant to drugs previously administered, which could increase the risk of relapse (32). Intensification therapies used include high doses of methotrexate and mercaptopurine, pulses of vincristine and glucocorticoids, and asparaginase for 20 to 30 weeks (29). In some cases, there is a reintroduction of the same treatment strategies used during the induction of remission phase.

After the consolidation phase, patients need a maintenance treatment to avoid relapse. Maintenance therapy is generally longer than the previous phases, with a duration between 24 and 36 months or longer (32). The regimen is composed by daily oral mercaptopurine or thioguanine and weekly oral methotrexate (106,107). In some cases, occasional pulses of glucocorticoids and vincristine is added.

The most common cause of treatment failure is relapse disease. In adults, about 60% of the cases will relapse (106). In pediatric, relapsed disease can occur as a result of isolated BM relapse, which occur in about 50% of the cases, CNS disease (accounts about 20% of the cases) and testicular disease (5% of the cases) (107). Reintroduction of intensified cytotoxic chemotherapies are used as salvage therapy which usually includes intensified doses of vincristine, corticosteroids and asparaginase (115).

High-risk patients with persistent relapses can be enrolled to allogeneic hematopoietic stem cell transplantation (28). This is the most intensive type of therapy for ALL and can be beneficial for high risk groups including patients with Ph-positive, high WBC count, CNS disease, high-risk gene rearrangements and individuals that do not respond to initial remission phase (28–30).

The standard therapeutic approaches have high rates of success. However short and long-term adverse effects are an issue to solve. A better understanding of the genetic and molecular alterations and pathogenic characterization of each individual will allow a direct and personalised therapy (28). Recent findings showed that good therapeutic responses can be achieved by inhibiting specific molecular targets essential for leukemogenesis, such as tyrosine kinases, DNA methyltransferase, histone deacetylase and proteosomes (36).

Monoclonal antibodies targeting specific antigens are becoming an important therapeutic tool to fight leukemia. CD22 is an antigen specific of B-lineage differentiation expressed in 50-100% and 90% of adult and pediatric B-ALL cases, respectively (116,117). Epratuzumab and Inotuzumab ozogamicin, which are monoclonal antibodies targeting CD22, can be conjugated with cytotoxic compounds such as calicheamicin that induce double-strand DNA breaks activating apoptotic mechanisms (118).

Besides monoclonal antibodies, proteasome inhibitors such as bortezomib are also reliable weapons. Bortezomib conjugated with vincristine, dexamethasone and

doxorubicin caused a response rate of 80% in B-cell ALL relapsed cases (119). Moreover, bortezomib is being studied since can inhibit signalling pathways as NOTCH1 and be used as therapy in T-cell ALL (28).

Hypomethylating agents such as decitabine inhibits DNA methyltransferase, promoting degradation and consequently hypomethylation of regulatory DNA domains (120). Dysregulation DNA methylation is associated to the development of ALL (due *DNMT3A* mutations, for instance) and its inhibition by hypomethylating agents can suppress leukemogenesis (121).

The involvement of multiple signalling pathways in development of ALL, including JAK-STAT pathway, has been reported (79,122). This pathway is responsible for maintenance and control of important developmental processes including cell growth, proliferation, and differentiation (79). Dysregulation of JAK-STAT found in B-ALL and T-ALL, is mainly because of genetic alterations that occur in key components of this pathway such as *IL7R*, Janus kinases (*JAK1, JAK2, JAK3* genes), negative regulators (*SH2B3*) and signal transducer and activator of transcription 5 (*STAT5*) (81,122,123). These alterations are usually gain-of-function mutations that contribute for abnormal activation of JAK-STAT signalling. Consequently, constitutively activation of this pathway leads to an uncontrolled proliferation of lymphoblasts (79). Thus, patients that harbor mutations in genes involved in JAK-STAT signalling, high-risk associated lesions, can benefit from inhibition of this pathway with therapeutic agents such as ruxolitinib (*JAK1* and *JAK2* inhibitor) in combination with other agents (49,124).

1.4. JAK-STAT pathway

The Janus kinase and signal transducers and activators of transcription (JAK-STAT) signalling pathway regulates hematopoiesis, including the formation of lymphoid cells (82). This cascade is responsible for signal transduction from the cell surface to the nucleus to control gene expression (125). The pathway is activated by cytokines, interleukins (IL) and growth factors, that regulate processes including cell proliferation, differentiation and apoptosis (125). Mutations that abnormally activate this pathway have been reported in high-risk B-ALL cases (Ph-like and Down-syndrome) and in T-ALL cases (49,122,126).

JAK-STAT comprise two main proteins responsible for signal integration, the Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs). In mammals, the JAK family is formed by four kinases, JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2) (127). These molecules comprise seven defined JAK homology domains (JH): from the carboxyl terminus (JH1) to the amino terminus (JH7) (**Figure 11**).

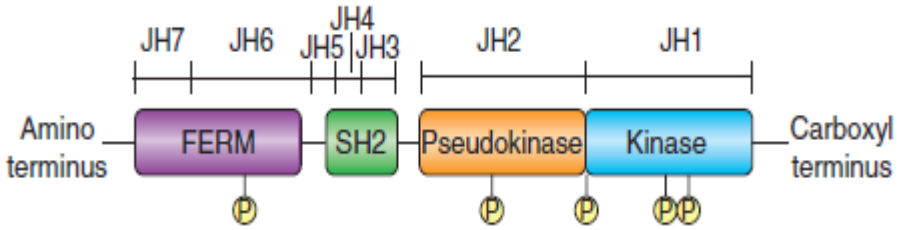

Figure 11 - Domain structure of Janus kinases (JAK). JAK proteins have 4 distinct domains, namely FERM, Src-homology-2 (SH2), Pseudokinase and Kinase domains. FERM domain is responsible for binding to the cytokine receptor and for regulation of catalytic activity. Pseudokinase domain also regulates catalytic activity and interacts with kinase domain. Autophosphorylation (P) occur in FERM, Pseudokinase and Kinase domains, and leads to the conformational changes of the JAK protein to facilitate substrate binding (Adapted from Yamaoka *et al.* 2004 (127)).

The JH1 domain contains characteristics of a tyrosine kinase and expresses catalytic activity. JH2 domain is a pseudokinase catalytically inactive and behaves as a regulatory portion. JH3, JH4 and JH5 share homology with Src-homology-2 (SH2) domain. JH6 and JH7 are present in the amino terminal (also called FERM domain) which is important for receptor recognition and binding. While JAK1, JAK2 and TYK2 are ubiquitously expressed, JAK3 is predominantly expressed in hematopoietic cells where is involved in regulation of cell development (128). Moreover, JAKs are selectively and intrinsically associated with their cytokine receptors located in the cell membrane (127,129).

The STAT family is composed by seven members STAT1, STAT2, STAT3, STAT4, STAT5 (STAT5A and STAT5B), and STAT6 (130,131). These proteins share six different domains with distinct functions (**Figure 12**). The N-terminal domain is responsible for STATs dimerization and binding to adjacent DNA sites. The core fragment is constituted by four domains: 1) Coiled-Coil Domain (CCD), responsible for nuclear receptor binding and, consequently, nuclear importation and exportation; 2) DNA Binding Domain (DBD), responsible for importin binding and for the specificity of STAT-DNA recognition; 3)

Linker Domain (LD), important for DNA-binding motif structure and for nuclear exportation of STATs in inactive cells; 4) SH2 domain, responsible for receptor dimerization. The C-terminal domain is involved in the activation of transcription through mobilization of transcription proteins. STATs are usually found "inactive" in the cytoplasm (132).

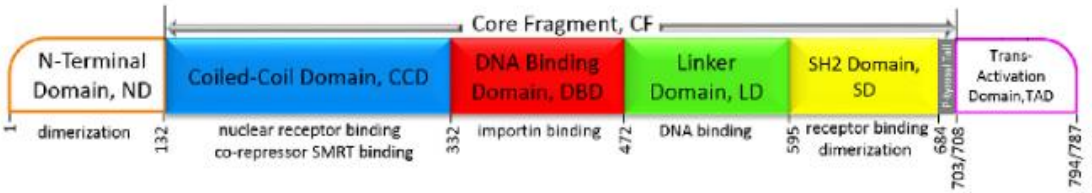

Figure 12 - STAT protein structure. STATs have multiple domains: a N-terminal domain (ND), a coiled-coil domain (CCD), a DNA binding domain (DBD), a linker domain (LD), a SH2 domain (SD) and a C-terminus domain composed by a trans-activation domain (TAD) (Adapted from Langenfeld *et al.* 2016 (131)).

The JAK-STAT activation process is initiated when ligands (e.g. cytokines) bind to the receptor, leading to receptor dimerization and, therefore, inducing conformational changes in the cytoplasmatic part of it (**Figure 13**) (125,129). The latter promote the proximity of JAKs located in each arm of the receptor, allowing transphosphorylation and, consequently, the activation of them. Moreover, active JAKs phosphorylate specific tyrosine residues that create a binding place to STATs. After STATs recruitment and phosphorylation, they translocate into the nucleus to regulate gene expression. The specificity of response is granted by different patterns of activated JAK and STAT proteins (125,133,134).

Besides the principal components of this pathway, the JAKs and the STATs, other molecules were identified to be important in the signalling. Effector proteins including signal-transducing adapter molecules (STAMs) and STAT-interacting protein (STIP) are important for transcriptional activation of specific target genes and for mediation of STATs phosphorylation by JAKs, respectively (125,135). STAM1 and STAM2A are associated with JAK2 and JAK3, upon cytokine signalling (135).

JAK-STAT pathway is also controlled by negative regulators such as suppressors of cytokine signalling (SOCs), protein inhibitors of activated STATs (PIAS) and protein tyrosine phosphatases (PTPs), responsible for inactivation of this pathway (**Figure 13**) (125,136). SOCs family is constituted by SOCS1 to SOCS7 and CIS, each of one composed

by a central SH2 domain, N-terminal domain of variable length and sequence, and C-terminal domain with SOCs box, a conserved region that facilitates proteolysis of STATs and JAKs (including their associated receptors) (137).

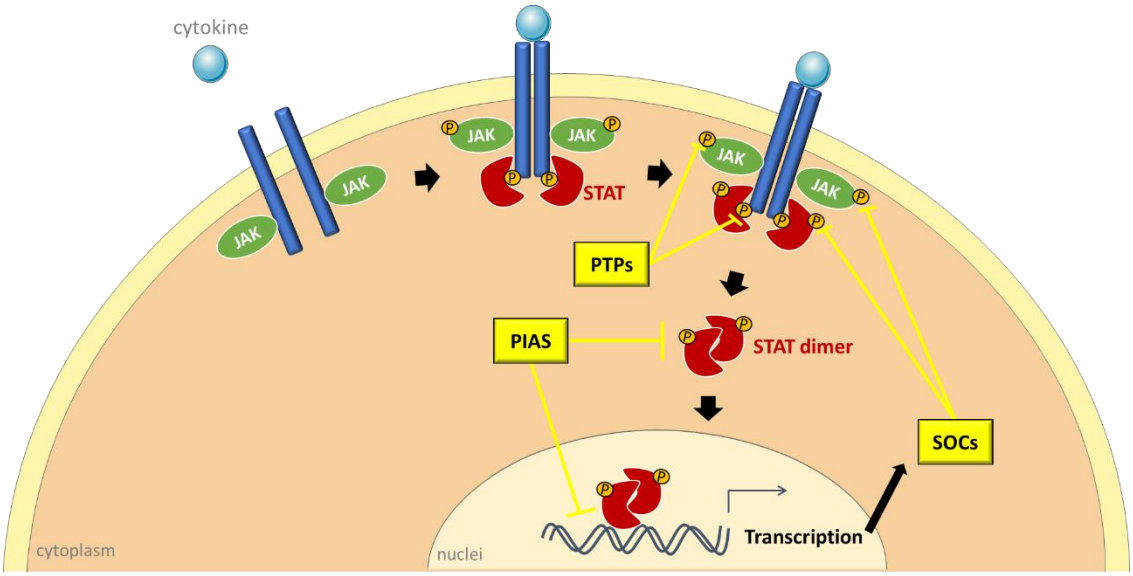

Figure 13 - Activation and regulation of JAK-STAT signalling pathway. Activation of this pathways is initiated when ligands (e.g. cytokines) bind to the receptor, inducing dimerization and conformational changes in the cytoplasmatic part of it. As consequence, Janus kinases (JAK), which are bound to the receptor, become closer to each, allowing phosphorylation of the receptor. This process attracts the Signal Transducers and Activators of Transcription (STATs). These proteins become phosphorylated and dimerize, further migrating into the nucleus. The STAT dimer binds to DNA and activate gene transcription. This pathway is also negatively regulated by suppressors of cytokine signalling (SOCs), protein inhibitors of activated STATs (PIAS) and protein tyrosine phosphatases (PTPs). SOCs can bind either to JAKs or to receptor inhibiting STAT recruitment. PIAS bind to STAT dimers preventing them from binding to DNA. PTPs can bind to phosphorylated receptor or JAKs to promote dephosphorylation of these proteins (figure based on Rawlings *et al*. 2004 (125)).

SOCS proteins are expressed by genes that are transcribed by activated STATs. Newly synthesized SOC proteins bind to JAKs, inactivating the receptors and consequently the pathway through a negative feedback loop. While SOCS1 interact directly with active JAKs through binding to its phosphorylated domain, SOCS3 requires the interaction with activated receptors and with activated JAKs, to have the same inhibiting effect. Additionally, CIS inhibitory function occurs by blocking STAT5 recruitment and phosphorylation. The PIAS proteins are constituted by PIAS1, PIAS3, PIASx and PIASy, which are composed by a central region with a Zn-binding RING-finger domain, a conserved N-terminal domain called SAF-A/Acinus/PIAS (SAP), and a less-conserved C-terminal domain (138). These proteins are constitutively expressed in cells

and functions as negative regulators by binding to activated STAT dimers, physically blocking active STATs translocated to the nucleus from binding to DNA (138).

Similar to SOCs, PTPs are negative regulators that inhibit JAK-STAT pathway by biding to activated receptor kinases or JAKs (139). SHP-1 and CD45 are two members of PTPs family. SHP-1 has two SH2 domains that can bind to phosphorylated tyrosine receptor or JAKs, promoting dephosphorylation of these proteins. CD45 is involved in hematopoiesis by regulating T and B cell receptor signalling which is important for lineage development (140). Posteriorly, has been found that CD45 regulates also JAK-STAT cytokine signalling by dephosphorylating JAK2 kinase, diminishing signalling activity (141).

The activation of JAK-STAT is started with activation of JAKs by binding a ligand (growth factors, interferons, or interleukins) to the specific receptor (132). Different types of receptors that are activated by those ligands include G protein-coupled receptors, receptor tyrosine kinases, homodimeric hormone receptors and cytokine receptors (132). Activation of JAK-STAT through cytokine receptors is made by a wide range of combinations of different JAKs and STATs units that influence signalling response (142). In fact, there is an intrinsic relationship between each cytokine receptor and the specific JAK that is activated. Because these receptors lack of kinase domain, JAKs are responsible to transmit the signal downstream to STATs (142). STATs are usually inactivated in the cytoplasm and recruited by JAKs that phosphorylate them in the C-terminal tyrosine residue. After phosphorylation, STATs assemble into homodimers or heterodimers and translocate into the nucleus, though nuclear importins, influencing gene transcription (142). STATs are not only activated by JAKs, they can integrate signals from other signalling pathways besides JAK-STAT and regulate diverse genes involved in cell proliferation and survival (132). Additionally, have been reported the role of JAKs in activation of other signalling pathways in other hematologic malignancies (143).

The JAK-STAT pathway is able to “crosstalk” with another signalling pathways (125). The crosstalk is mediated by activated JAKs that interact with adapter and effector proteins from other signalling pathways. Thus, JAK-STAT can crosstalk with RAS-RAF-ERK-MEK, PI3K-AKT-mTOR and FAK-MEK signalling pathways, influencing cell

proliferation, survival, cell cycle progression and promoting/repressing apoptosis (**Figure 14**) (144–146).

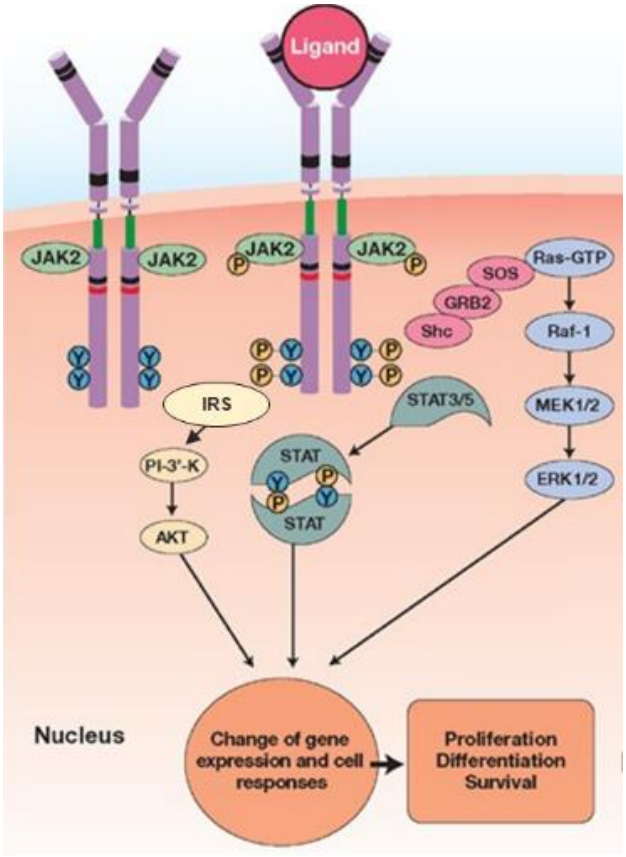

Figure 14 – JAK-STAT can crosstalk with another signalling pathways. Ras signalling is initiated by activated JAKs that create a binding site on their receptors for adapter proteins which are recruited (SHC, GRB2 and SOS). These proteins interact with RAS-GTP and stimulate RAS signalling. Moreover, JAKs can also stimulate PI3K cascade through recruitment and phosphorylation of insulin receptor substrate (IRS). Activation of these pathway are associated with proliferation, differentiation, and survival. (Adapted from Vainchenker *et al*. 2013 (82)).

Ras signalling is activated not only by growth factors and cytokines, but also by kinases including JAKs. The activation of RAS signalling occurs when active JAKs recruits adaptor proteins, including SH2-domain containing protein (SHC), growth factor-bond protein 2 (GRB2) and son of sevenless (SOS), that phosphorylates RAS promoting activation of RAF (125). These molecules activate the cascade through MEK/ERK promoting cell proliferation, differentiation, survival and inhibition of apoptosis (79). RAS cascade is frequently abnormally activated in cancer due activation by epidermal growth factor receptor (EGFR) which is intimately associated with JAK1 and JAK2 (132,144). Moreover, mutations in components of RAS pathway have been associated

as cooperating lesions in ALL development (147). JAKs can also activate PI3K-AKT-mTOR pathway by phosphorylating the insulin receptor substrate 1 or 2 (IRS 1/2), which results in activation of PI3K cascade (125,145). This pathway is important for regulation of cell growth, proliferation, differentiation and survival (by inhibition of apoptosis) (79). Moreover, PI3K pathway can be activated by growth factors and cytokines that bind to tyrosine kinases (RTKs) or G protein-coupled receptors (GPCRs) activating these receptors. Afterwards, PI3Ks are activated being recruited by the receptors, and activate AKT that phosphorylates certain proteins that regulate gene transcription. Constitutive activation of this pathway is associated with leukemic development (70).

In this manner, the mechanism of JAK-STAT signalling is dependent of JAKs and STATs activation. However, cellular response can reflect side activation of additional signalling pathways.

1.4.1. Dysregulation of JAK-STAT

Molecular alterations that induce an arrest of differentiation are responsible for dysregulation of important cellular processes, including cell proliferation and survival, being associated with leukemic transformation of lymphoid progenitors. These alterations are intimately connected with dysregulation of important signalling pathways as JAK-STAT. The development of new techniques such as next-generation sequencing (NGS), allowed the identification of new genetic alterations involving JAK-STAT cascade (79). Somatic mutations involving genes associated with B-cell development and differentiation are found in about 11% of B-ALL cases. In these cases, are frequently found mutations involving *JAK1*, *JAK2*, *IL7R* and *CRLF2* genes (126,148). Regarding T-ALL, JAK-STAT signalling associated lesions are found in about 20-30% of the cases. Mutations in *JAK1*, *JAK2*, *JAK3*, *IL7R* and *STAT5B* are usually reported in these cases (80,122). These genetic alterations are associated with high-risk ALL cases, including Ph-like ALL, Down Syndrome-associated B-ALL and T-ALL (49,122,126,149).

Several kinase activating alterations are found in patients with Ph-like ALL (49). These patients have identical gene-expression profile to Ph-positive but do not express *BCR-ABL1* fusion gene (25). Rearrangements of cytokine receptor like factor 2 (*CRLF2*) gene associated with *JAK1* or *JAK2* mutations are frequently detected Ph-like cases,

being associated with worst outcome (49). Moreover, sequence mutations and deletions affecting JAK-STAT signalling are identified in additional components, including in interleukin-7 receptor (*IL7R*), in fms-like tyrosine kinase 3 (*FLT3*), which is a receptor tyrosine kinase, and in SH2B adaptor protein 3 (*SH2B3*), a negative regulator of JAK2 signalling (49,148). These alterations promote aberrant activation of JAK-STAT signalling independently of cytokine activation. Additionally, it has been reported the activation of PI3K-mTOR signalling in B-ALL cases with *CRLF2* rearrangement with concomitant *JAK2* mutations evidencing the crosstalk between JAK-STAT and PI3K signalling (52).

Patients with Down syndrome (DS) are associated to high-risk B-ALL, as a result of poor survival rates and high prevalence of relapse (126). These patients exhibit chromosomal rearrangements leading to the aberrant expression of CRLF2 that heterodimerizes with IL7R, and shape into thymic stromal lymphopoietin (TSLP) receptor (126,149). The activation of TSLP receptors stimulates the JAK-STAT pathway. Moreover, further activating mutations involving JAK-STAT occur frequently in *CRLF2*, *IL7R, JAK1* and *JAK2* genes (126). Thus, *CRLF2* rearrangements and additional activating mutations enhance JAK-STAT signalling in DS-ALL (126,149).

The proliferation and survival capacity of lymphoblasts result from aberrant activation of signalling pathways implicated in normal T-cell development including JAK-STAT. During T-cell development, JAK-STAT signalling is activated through interleukin 7 (IL7), promoting cell survival and cell cycle progression (122). ILR7 is activated by IL7, which results in dimerization of the receptor, and consequently transphosphorylation of JAK1 and JAK3. JAKs recruit and phosphorylate STAT5, that dimerizes and translocate to the nucleus to regulate gene transcription. This signalling is crucial for T-cell maturation in the thymus.

ETP-ALL which is as subtype of T-ALL that exhibit a block at the early stages of thymocyte differentiation, has a high prevalence of mutations involving JAK-STAT signalling genes (80,122). Activating mutations in *IL7R* are found in about 10% of these patients (83). Additionally, activating mutations in *JAK1*, *JAK3* and *STAT5,* and a rare t(9;12)(p24;13) translocation encoding for ETV6-JAK2 fusion gene, that lead to aberrant activation of JAK-STAT signalling, are less frequently found in T-ALL patients (85,86,150,151).

Usually, aberrant activation of STAT5 in ALL can occur as a consequence of abnormal JAKs activity generated by mutations or rearrangements involving these kinases. However, gain of function mutations in *STAT5B* have reported as being divers of leukemic transformation (86). These mutations occur mainly in SH2 domain of *STAT5*, promoting constitutive signalling, and are associated with poor outcomes and high risk of relapse (152). Moreover, constitutively activated STAT5 show high DNA binding activity detected in leukemic cells of Ph-ALL patients (153). This evidence can be a prove that STAT5 is an effector of BCR-ABL fusion protein. Additionally, dysregulation of STAT5 activity is linked to drug resistance, pointing it as a potential therapeutic target in ALL (154).

1.5. Calreticulin

Calreticulin (CALR) is an important protein for many cellular functions including regulation of Ca^{2+} homeostasis, protein folding, gene expression and immune response (155).

In terms of structure, calreticulin is a 46 KDa protein and has three domains, the N-domain, P-domain and C-domain (**Figure 15**) (156). The N-terminal is a globular domain and is highly conserved among species (155,156). This domain contains a cleavable signal and a disulphide bond established by cysteine residues that is thought to interact with P-domain and together play a role in the protein's chaperone function. The P-domain is proline rich and include repetitive regions that form structures of lectin-like chaperone, which are involved in protein folding functions (155). This domain has high affinity but low capacity to bind Ca^{2+}. Finally, C-domain is important for calcium buffering since it has high capacity but low affinity for Ca^{2+}, as well as for calreticulin translocation from the endoplasmic reticulum (ER) lumen to the cytoplasm due to the presence of the amino acid sequence KDEL (155). In addition, Ca^{2+} binding to this region promotes interaction with other chaperone proteins in the ER (155,157,158).

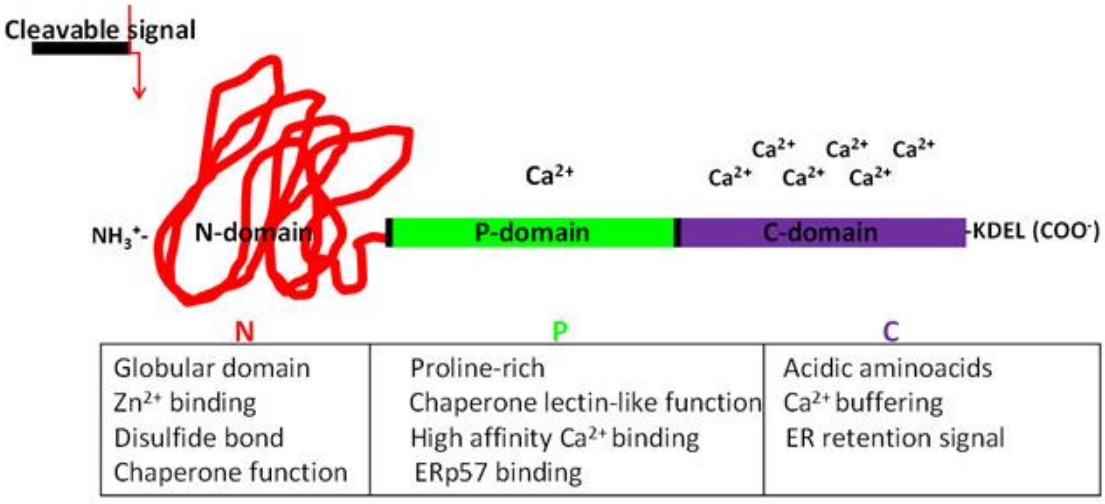

Figure 15 - Structure and functions of Calreticulin domains. Calreticulin is a calcium (Ca^{2+}) binding chaperone that plays an important role in quality control processes during protein synthesis and folding. This protein is mainly located in the endoplasmic reticulum (ER) and has three domains with different functions. The amino (N) domain has a signal sequence that once cleaved, calreticulin is transported into the ER following synthesis. P domain contains a high-affinity Ca^{2+} binding site and lectin-like chaperone site. Thus, calreticulin is involved in Ca^{2+} metabolism and protein-protein interactions, namely glycoproteins interactions. Finally, the carboxyl (C) domain is important in determining the Ca^{2+} storage capacity of the ER, thus allowing Ca^{2+} binding. This domain has a KDEL signal, constituted by an amino acid sequence of lysine (K), aspartate (D), glutamate (E) and leucine (L), that prevents calreticulin from being secreted from the ER (Adapted from Mendlovic *et al*. 2011 (158)).

CALR is involved in important cellular processes, not only under physiologic conditions (**Figure 16A**) but also in pathologic circumstances (**Figure 16B**) (159).

This protein is found in the ER being responsible for protein chaperoning and regulation of Ca^{2+} homeostasis (**Figure 16A**) (157). Here, CALR interact directly with newly synthesized glycoproteins being responsible for their correct folding and functional shape before the secretion to their specific locations (155). Most of the intracellular Ca^{2+} is stored in ER and CALR is associated with about 50 % of that storage, due its capacity of binding Ca^{2+} (160). Ca^{2+} is an important signalling molecule and activation of transcriptional and translational cascades is dependent on Ca^{2+} levels (155). Alterations in ER luminal Ca^{2+} levels influence cellular processes including protein trafficking between ER and Golgi apparatus, transport of molecules across the nuclear pore and protein folding (156,161,162).

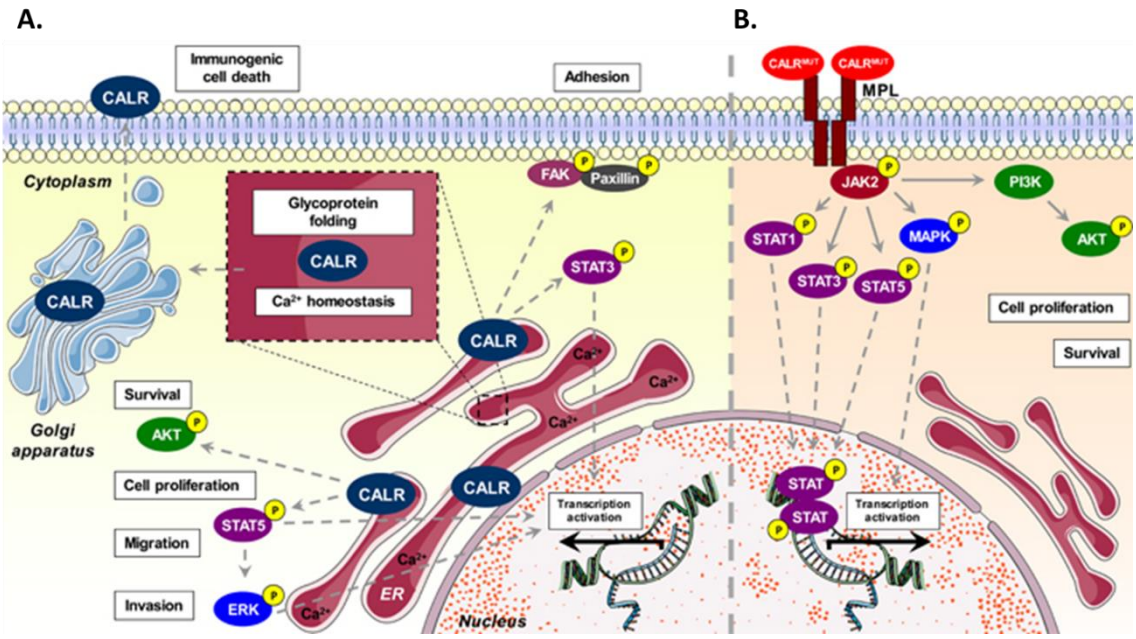

Figure 16 – Physiologic and pathologic roles of CALR. A. Within endoplasmic reticulum (ER), CALR is responsible for the correct folding of synthesized glycoproteins, and regulation of Ca^{2+} storage and releasing. Under stress conditions, CALR is translocated to the cell surface through exocytosis vesicles and participates in immunogenic cell death acting as a phagocytosis signal. Moreover, CALR regulate focal adhesion of cells through focal adhesion kinase (FAK) signalling. Additionally, CALR is important for control of cell proliferation, survival, migration and invasion. CALR regulates important signalling cascades, including PI3K-AKT and ERK signalling, through direct interaction with upstream signal transducers and activators of transcription (STATs). **B.** Under pathologic conditions, mutations involving CALR promote the transportation of mutated CALR to the cell surface. Here, they interact with thrombopoietin receptor which dimerizes and activate downstream JAK2. On his turn, JAK2 activated STATs, MAPK and PI3K-AKT signalling which promotes an increase in cell proliferation and survival (Adapted from Machado-Neto *et al.* 2018 (159)).

Besides ER, CALR can be found in the cell surface where is involved in cell adhesion and immune response (157). CALR has an important role in regulation of extracellular matrix molecules (ECM) expression, affecting cell adhesion processes (163). Knockdown studies revealed that depletion of CALR in cancer cell lines, resulted in significantly reduced growth rate and cell motility *in vitro*, indicating that CALR is necessary for cell proliferation and migration (163). Additionally, CALR regulate focal adhesion of cells and activity of matrix metalloproteinases. Thus, focal adhesion kinase (FAK) was downregulated in CALR-knockdown cells and the phosphorylation levels of paxillin (a focal adhesion protein) decrease, suggesting the involvement of FAK pathway.

Moreover, reduction of CALR levels decreases the migration and invasion capacity of esophageal carcinoma cells (164–166). CALR promotes the transcription of genes related with migration and invasion of these cells which is mediated by activation of

STAT3 and STAT5, and therefore PI3K-AKT cascade (164,165). Additionally, knockdown of CALR revealed a connection between CALR, STAT5A and ERK in the regulation of esophageal carcinoma cells migration (166).

In hepatocellular carcinoma cells, downregulation of CALR inhibits cell proliferation and invasion, induces apoptosis and arrests cell cycle progression (167). Moreover, knockdown of CALR inactivate PI3K-AKT pathway by suppressing phosphorylation of AKT, suggesting that CALR is the upstream signal of PI3K-AKT cascade.

Additionally, expression of CALR on the cell surface induces immune response and apoptotic cells clearance by phagocytosis (168). Has been reported that chemotherapeutic therapies with anthracyclines frequently used against cancer, including ALL, induce high levels of CALR expression on the cell surface, promoting phagocytosis-mediated cell death (169). However, these cells display also high expression levels of CD47, which has a opposite function of CALR and is a signal to prevent phagocytosis (170).

CALR alterations cause impairment of its normal functions and promote pathogenesis (**Figure 16B**). Mutations in *CALR* gene are usually deletions or insertions in the last exon (in exon 9) that create a mutant CALR with a shorter or longer C-domain, lacking KDEL motif (171–173). Consequently, this protein has a different cellular localization since is not retained in the ER. Moreover, these mutations alter C-terminal domain by replacing negatively charged amino acids by a positively charged, which impair Ca^{2+} binding function (172). Despite the fact that *CALR* alterations occur early development in cells with myeloid and lymphoid differentiation capacity, they are expressed frequently in myeloid lineages (174). Thus, *CARL* mutations are reported in Ph-chromosome-negative myeloproliferative neoplasms (MPN), including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) (172,173,175,176). In these cases, CALR mutations abnormally activate JAK-STAT pathway independently of cytokine binding (**Figure 16B**) (177,178). Mutant CALR binds to thrombopoietin receptor (MPL) and induces JAK2 phosphorylation activating it. A report where it was induced homologous mutations in the murine *CALR* gene, which expressed a highly similar to humans CALR-mutant protein, this protein activated constitutively JAK-STAT signalling through JAK2 (179). Further downstream of JAK2,

occurs activation of PI3K-ATK cascade and STATs, including STAT1, STAT3, and STAT5 (172,179). Thus, mutant CALR induces activation of JAK-STAT/PI3K-AKT signalling pathways, influencing cell proliferation and survival.

Patients with essential thrombocythemia and primary myelofibrosis were found to carry *CARL* mutations, becoming the second most frequent mutation after *JAK2* (172). *CALR* mutations are mutually exclusive with the most frequent mutations occurring in *JAK2* and *MPL* genes (172,173). Between 70 % and 84 % of the patients exhibit *CALR* mutations but lack in mutations of JAK2 and MPL (172,173). However, these patients have longer overall survival than those with *JAK2* mutations (172). Mutant calreticulin lacks in KDEL signal and translocate to the cell surface being responsible for activation of MPL and JAK-STAT signalling.

While the role of *CALR* mutations in MPN is well established, in acute lymphoblastic leukemia is less known. To date, no mutations were found in patients diagnosed with ALL (173,175). However, has been reported development of second leukemias arising from MPN, including B-ALL and T-ALL (180,181). In one case of ET, cell sorting and mutational analysis of HSCs and progenitor cells, detected a *CALR* mutation in the lymphoid lineage (181). The malignant transformation of ET into ALL can be derived from a subclone earlier to the acquisition of the CALR mutation. Thus, this is the first report of a B-ALL arising in a patient diagnosed with ET and harbouring a *CALR* mutation (181).

Additionally, *CALR* can be a potential prognostic gene for ALL (182,183). Based on mRNA expression profile and survival analysis, *CALR* was identified as a potential biomarker for ALL, which can be used for risk stratification and development of new therapeutic approaches (182,183).

1.6. JAK-STAT inhibitors: Ruxolitinib and Pimozide

JAK-STAT is an essential pathway involved in distinct cellular processes including survival, proliferation, and differentiation. Chromosomal rearrangements and activating mutations including those in *JAK1, JAK2* and *JAK3*, and *STAT5,* are genetic alterations that contribute to dysregulation of JAK-STAT signalling in ALL. These alterations

frequently involving JAKs and STATs generally activate constitutively JAK-STAT signalling, resulting in uncontrolled proliferation and survival of leukemic cells. Although low incidence of JAK-STAT genetic lesions, they are associated with high-risk ALL and poor outcomes. Thus, this pathway is an opportunity and attractive therapeutic target to molecular inhibitors including TKIs and STAT inhibitors.

Ruxolitinib is a specific inhibitor of the JAK1, JAK2 and JAK3 (being more selective for JAK2), approved as a therapeutic drug for the treatment of myelofibrosis in 2011 by the Food and Drug Administration (FDA) (**Figure 17A**) (184). This inhibitor has shown therapeutic efficacy not only in myeloproliferative neoplasms but also in Ph-like ALL (124,185,186). This ALL subtype is characterized by high relapse frequency and therapeutic resistance (185,186). Usually, these patients exhibit alterations involving JAK-STAT pathway, including *CRLF2* rearrangements and concomitant *JAK1* and *JAK2* mutations leading to aberrant activation of the pathway. Additionally, about 20% of these patients show *JAK2* rearrangements which activate JAK-STAT signalling (185). Ruxolitinib has shown clinical efficacy in Ph-like ALL with *GOLGA5-JAK2* rearrangement after induction failure (186). Administration of high-doses of ruxolitinib in combination with multi-agent chemotherapy resulted in reduction of leukemic burden as well as *GOLGA5-JAK2* fusion levels, evidencing clinically efficacy of this combination. However, the patient demonstrated levels of MDR detected by flow cytometry which required additional therapies (186). Moreover, ruxolitinib in combination with intensive multi-agent chemotherapy induces a dose-dependent inhibition of phosphorylated STAT5 in Ph-like children and adolescents/young adults (AYAs) (185). In addition, administration of ruxolitinib in patients that harbour *JAK2* mutations has demonstrated a significative decrease in lymphoblasts (187).

The involvement of central nervous system is responsible for about 10 % of ALL relapses due limited penetration of chemotherapeutic drugs through the blood-brain barrier (BBB) (188). Ruxolitinib in combination with IT therapy (methotrexate, cytarabine and hydrocortisone) has shown efficacy in penetrating the BBB and eliminating highly refractory leptomeningeal B-ALL (189).

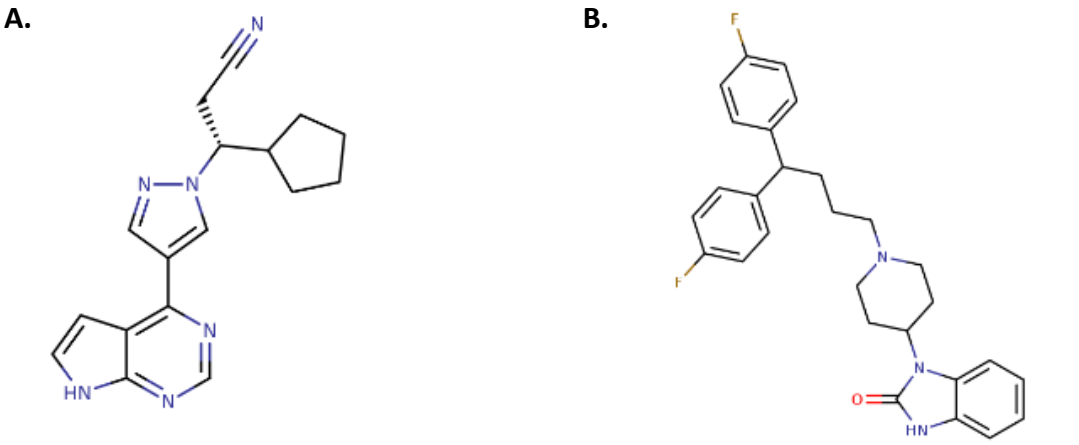

Figure 17 - Inhibitors of the JAK-STAT pathway. A. Molecular structure of Ruxolitinib and **B.** Pimozide (Adapted from PubChem Database (231,232)**.**

STAT5 is a downstream effector of JAK2. Constitutive activation of this transcription factor is usually associated with dysregulated JAK2 activity. However, genetic alterations found in *STAT5* are also responsible for abnormal activation of these proteins which leads to transcription of target genes involved in cell proliferation and survival (86,152).

Pimozide is an active psychotropic agent that is very effective blocking dopaminergic receptors of neurons in the nervous system, decreasing dopamine neurotransmission (**Figure 17B**) (190). The use of this drug was approved in treatment of neuropsychiatric diseases including schizophrenia and Tourette´s Disorder (191,192). Besides its neurologic activity, Pimozide was identified as a STAT5 inhibitor displaying an antileukemic effect in hematological malignancies (193,194). Pimozide is effective as antiproliferative drug in chronic myelogenous leukemia (CML) cells that are resistant to kinase inhibitors (193). Pimozide induces a reduction of STAT5 phosphorylation as well as expression of STAT5 target genes involved in survival and growth of CML cell lines. Moreover, pimozide reduces CML cell lines viability as a result of induction of apoptosis and cell cycle arrest in these cells, at G_1 phase. Additionally, combination of pimozide with TKIs, imatinib and nilotinib, results in enhanced inhibition of STAT5 phosphorylation which impacts leukemic proliferation (193).

In accordance with the results obtained in CML cell lines, pimozide demonstrates similar results in other MPN cells (195). Treatment of MPN cell lines with pimozide induce a decrease in tyrosine phosphorylation of STAT5. This inhibition can be a result

of the direct interaction of pimozide with STAT5 or with a negative regulator of STAT5. Moreover, inhibition of STAT5 phosphorylation influence the expression of STAT5 target genes related with cell survival, including B-cell lymphoma-extra-large (*BCL-XL*) and myeloid cell leukemia sequence 1 (*MCL1*). Additionally, pimozide affect the viability of MPN cell lines, inducing cell cycle arrest and apoptosis.

The combination of pimozide with a JAK2 inhibitor enhanced the effect of pimozide alone (195). Cells treated with the combination showed a near-complete loss of STAT5 phosphorylation, a greater reduction in the level of pro-survival Mcl1 protein, and a greater decrease in cell viability, suggesting an enhanced cytotoxicity induced by this combination in MPN cells (195).

1.7. Aims

ALL is a hematological malignancy classified in multiple subtypes, which are characterized by genetic alterations that establish genetically distinct groups. The identification of structural aberrations has been important for the establishment of correct diagnostic, risk determination and development of target therapies. While the vast majority of ALL patients enter in remission after induction phase of conventional therapeutic schemes, a small portion relapse (28). Thus, efforts have been made to identify new molecular targets involved in leukemic transformation and develop new therapeutic agents.

The involvement of JAK-STAT signalling pathway in development of ALL is well reported (82,85–87,126,148,154). About 11 % of B-ALL, and between 20 and 30 % of T-ALL cases, exhibit mutations involving *JAK1, JAK2, IL7R, CRLF2* and *STAT5* genes (126,148). Additionally, chromosomal translocations such as *ETV6-JAK2* and *BCR-ABL* can influence direct- and indirectly JAK-STAT signalling (87,154). These genetic alterations activate aberrantly JAK-STAT signalling which promote subsequently dysregulation of the expression of target genes involved in leukemic proliferation and survival. Targeting the JAK-STAT pathway could be an interesting approach for therapy, since it could decrease the constitutive activation of the signalling pathway and prevent proliferation and survival of leukemic cells.

From this perspective, the aim of this work is to characterize and evaluate the therapeutic potential of two JAK-STAT pathway inhibitors, Ruxolitinib and Pimozide, *in vitro* ALL cell lines. We assessed the therapeutic potential of different schemes of administration, in monotherapy or in combination (Ruxolitinib and Pimozide).

Chapter 2 – Methods

2.1 Cell lines characterization

2.1.1 Cell lines and cell culture

In this study, five *in vitro* cell models of ALL were characterized and used to perform pharmacological assays: 697 and REH as B-cell precursor leukemia models, and CEM, JURKAT and MOLT4 as T-cell precursor leukemia models.

The 697 cell line was established from a bone marrow aspirate collected from a 12-years-old boy diagnosed with B-ALL at relapse in 1979 (196). Genetic studies identified a translocation between chromosome 7 and 19 (t(7;19) (q11;q13)), leading to expression of *TCF3-PBX* fusion gene (196). These cells exhibit upregulation of *BCL2*, *BLC3* and *MYC* mRNA.

The other model of B-ALL, REH cell line was established from a peripheral blood sample of a 15-years-old North African girl at first relapse. This cell line exhibited *TEL-AML1* (*ETV6-RUNX1*) fusion gene generated by translocation t(12;21)(p13;q22) (197,198).

The CEM cells were obtained from peripheral blood sample of a 4-years-old Caucasian girl with T-ALL at relapse (199). This cell line presented a novel t(5;14)(q35.1;q32.2) which encodes *NKX25-BCL11B* fusion gene (200). Morphologically these cells show features of precursor T-cell ALL, expressing T-surface antigens CD3, CD4, CD5 and CD7.

The JURKAT cell line was established from the peripheral blood sample of a 14-years-old boy with T-ALL at relapse in 1976 (201). Cytogenetically, these cells presented a karyotype hypotetraploid.

The MOLT4 cell line was established from the peripheral blood sample of a 19-years-old man with T-ALL at relapse, after multidrug chemotherapy. These cells have a karyotype hypertetraploid with 96 chromosomes. The P53 protein is not expressed, due to a missense mutation at codon 248 of the *TP53* gene (202).

697 cell line was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, German). REH, CEM, JURKAT and MOLT-4 cell lines were purchased from American Type Culture Collection (ATCC, USA). These cells were grown in suspension in *Rowell Park Memorial Institute* 1640 medium (RPMI-1640; Sigma-Aldrich,

USA) containing 2 mM L-glutamine and 20 mM HEPES-NA and supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, USA), 100 U/mL penicillin (Gibco, Invitrogen, USA), 100 µg/mL streptomycin (Gibco, Invitrogen, USA), at pH 7.4. Cells were cultivated under culture conditions at 37º C in a 5 % CO_2 humidified atmosphere.

2.1.2 Cell proliferation and cell viability assessment by Trypan Blue Exclusion Assay

Trypan blue exclusion assay allows to assess the cell proliferation and viability through determination of cell density and the number of viable cells (203). Viable cells have intact membranes that block the entrance of certain dyes such as trypan blue. On the contrary, death cells do not have intact membranes and are permeable to the dye becoming stained as blue.

All cell lines were cultured, following the conditions described in 2.1.1., for 72 hours at initial densities of 0.3 x 10^6 cells/mL, except REH cell line with an initial density of 0.5 x 10^6 cells/mL. At each 24 hours, a sample of cell suspension was collected and cells were counted with trypan blue (Sigma-Aldrich, USA) using an hemocytometer (Neubauer chamber). Cell viability was determined by dividing the number of viable cells, i.e. cells with a clear cytoplasm, for the total number of cells (**Equation 1**). In addition, cell density was assessed by **Equation 2**. Results represent the mean ± standard error of mean (SEM) of 3 independent experiments. 10^{-4}

$$Cell\ viability\ (\%) = \frac{total\ number\ of\ viable\ cells}{total\ number\ of\ cells}\ x\ 100 \qquad \textbf{(Equation 1)}$$

$$Cell\ density\ (10^6/mL) = number\ of\ viable\ cells/10^{-4} \qquad \textbf{(Equation 2)}$$

2.2 Evaluation of *CALR* gene expression and mutational profile

2.2.1 RNA extraction

The RNA of each cell line model was extracted using NZYol reagent (Nzytech, Portugal), according to manufacturer instructions. This reagent allows the sequential isolation of RNA, DNA and proteins from a sample and is composed mainly by phenol and guanidine isothiocyanate. When chloroform is added to NZYol, it occurs phase-separation: (i) the upper layer is aqueous and contains RNA; (ii) the interphase contains DNA; (iii) the lower layer (organic phase) contains proteins (**Figure 18**).

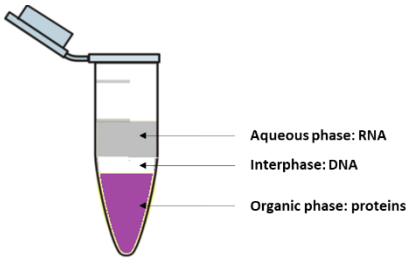

Figure 18 - Phenol-chloroform phase-separation of NZYol reagent. The aqueous layer contains RNA, the interphase contains DNA and the lower phase contains.

Briefly, 5 million of each cell line were washed with PSB by centrifugation at 200xg and resuspended in 1 mL of NZYol. After 5 minutes incubation at room temperature, 200µL of chloroform was added to the sample. Following 3 minutes incubation, the sample was centrifuged at 12.000xg for 15 minutes at 4ºC to separate the different layers. The aqueous phase was transferred to another microtube, where 500µL of isopropanol was added and the sample incubated for 10 minutes at room temperature. Then, it was centrifuged 12.000xg for 10 minutes at 4ºC to precipitate the RNA. The supernatant was removed and 1mL of ethanol added, followed by centrifugation. The pellet was dried in air to remove vestigial amounts of ethanol. Finally, RNA pellet was dissolved in DNase/RNase free water and incubated for 10 minutes at 55ºC. The RNA was stored at -80ºC for posterior use.

RNA yield was determined by quantification in NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). This method measures absorbance

(A) at 230nm, 260 nm and 280nm to assess RNA concentration (260/230 ratio) and purity (A260/A280 ratio) in the sample (204).

2.2.2 cDNA synthesis

RNA was converted to cDNA using GRS cDNA Synthesis Mastermix kit (Grisp, Portugal). Briefly, 2 µg of RNA template was diluted in RNase-free water and mixed with 1 µL of primers for total volume of 10 µL. After that, sample was heated for 5 min at 65ºC to remove secondary RNA structures and then placed 2 min on ice. To each sample was added 10 µL of GRS RT Mastermix. cDNA was synthesized in thermocycler (T100 Thermal Cycler, Bio-Rad, USA), for 10 min at 25ºC, followed by 30 min at 55ºC and 5 min at 85ºC. The cDNA was stored at -20ºC for later use.

2.2.3 Quantitative real time-polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) was performed to determine the expression levels of *CALR* in the six ALL models. For that purpose, *CALR* and *GUSB* (endogenous control) genes expression levels were measured using XPERT SYBR Green mastermix (GRiSP, Portugal), in a QuantStudio™ 3 System (ThermoFisher Scientific, USA).

To analyse the *CALR* gene expression were used 250nM of each primer and 200nM of primers in *GUSB* reaction. The sequences of *CALR* primers used to perform qPCR were: 5'-CCCTGAGTACAAGGGTGAGTG-3', as forward, and 5'-TTCTGGGTGGATCCAAGTGC-3', as reverse. The sequences of *GUSB* primers used were: 5'-CAGGTGATGGAAGAAGTG-3', as forward, and 5'-AAGTAGTAGCCAGCAGAT-3', as reverse. Standard curves of K562 and NB4 cDNA were used to determine the reaction efficiency of *CALR* and *GUSB*, respectively. The thermocycler parameters were the denaturation step at 95º C for 3 minutes, followed by 40 cycles of annealing and extension for 5 seconds at 95º C, and 25 seconds at 65º C. All samples were used in duplicate and no template controls were included.

The relative expression was determined using the ΔCt method using GUSB as a reference gene, according to the following equations:

$$\Delta Ct = CALR\ Ct - GUSB\ Ct \qquad \textbf{(Equation 3)}$$

$$Expression\ levels = 2^{\Delta Ct} \qquad \textbf{(Equation 4)}$$

The results represent mean ± standard error of the mean (SEM) of 3 independent experiments.

2.2.4 DNA isolation

DNA isolation was made using TRIzol ™ reagent (Invitrogen, USA), accordantly with manufacturer´s protocol. During phase separation, RNA is present in the aqueous phase, DNA in the interphase, and proteins and lipids are present in the organic phase. Therefore, DNA can be isolated from the interphase after removal of the overlying aqueous phase. Following the removal of the aqueous phase, 300 µL of ethanol 100 % was added to remaining solution containing the interphase and the organic phase. Then the tubes were mixed, incubated for 5 minutes at room temperature and centrifuged at 2.000xg for 5 minutes at 4ºC to *pellet* the DNA. To wash the DNA, the supernatant was discarded and the *pellet* was resuspended in 1 mL of 0.1 M sodium citrate in 10 % ethanol, at pH 8.5. The samples were incubated for 30 minutes at room temperature and occasionally inverted. Afterwards, the samples were centrifuged at 2.000xg for 5 minutes at 4ºC. The washing steps were repeated once. Then the *pellet* was resuspended in 1.5 mL of ethanol 70 % and the samples were incubated for 15 minutes at room temperature, being occasionally inverted. Subsequently, the samples were centrifuged at 2.000xg for 5 minutes at 4ºC and supernatant removed. The DNA *pellet* was air dried for enough time to remove the remaining ethanol. Finally, the DNA was solubilized in DNase/RNase free water and incubated for 10 minutes at 55ºC. Insoluble particles were removed from the sample by a centrifugation at 12.000xg for 10 minutes. The supernatant with DNA was transferred to a new tube and stored at -20ºC for posterior use.

The DNA yield was determined and quantified in NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA), where values of 1.8 represent a highly pure sample.

2.2.5 Sanger sequencing of CALR

To detect possible mutations in exon 9 of *CALR* gene, Sanger sequencing was performed. This technique determines the sequence of nucleotide bases using specific primers that bind to target DNA regions (205). The process starts when double-stranded DNA is denatured into two single-strands allowing the binding of oligonucleotide primers. Deoxynucleotide triphosphates (dNTPs) are essential to produce and elongate the new double-stranded chain while dideoxynucleotide triphosphates (ddNTPs) terminates this elongation when attached. To detect the sequence of nucleotide bases, each ddNTPs has a fluorescent marker bonded. This marker will fluoresce when a ddNTP (ddATP, ddGTP, ddCTP or ddTTP) binds to the new chain and the fluorescence is dependent of the nucleotide. Based on scientific agreement, each nucleotide has a different fluorescence associated: when adenine (A) is attached fluoresces green, cytosine (C) fluoresces blue, tyrosine (T) red and guanine (G) black. The new synthetized DNA sample is run through a laser that excites the fluorescent marker present in nucleotide bases and the fluorescent intensity is translated into an electropherogram that shows the fluorescent peak of each nucleotide.

The sequence of primers designed to detect possible mutations in exon 9 of *CALR* gene were: “CTTCCTCATCACCAACGATGA” as forward primer and “CACAGAGACATTATTTGGCGC” as reverse primer. Amplification of DNA samples was run during 98º C for 3 minutes, 95º C for 30 seconds to denaturate DNA strands, 60º C for 30 seconds for annealing, and 72º C for 30 seconds for fragment extension. This process is repeated for 34 cycles. Afterwards, samples were sequenced through Sanger Sequencing method in the 3130 Genetic Analyser (Applied Biosystems, USA).

2.3 Evaluation of the therapeutic potential of JAK-STAT inhibitors: Ruxolitinib and Pimozide

2.3.1 Cell culture and therapeutic schemes

Two JAK-STAT inhibitors were used in this study: ruxolitinib, which is a JAK1/JAK2 inhibitor, and pimozide which inhibits STAT5. ALL cell lines were cultured under the

same conditions as described in 2.1.1, and incubated in the absence and presence of increasing concentrations of Ruxolitinib (RUX), 2.5 µM to 25 µM, and Pimozide (PIM), 1 µM to 10 µM, for a period of 72 hours. Three therapeutic schemes were tested: (i) monotherapy as a single administration; (ii) monotherapy as daily administration; (iii) combination of both inhibitors (**Figure 19**). At each 24 hours of incubation, metabolic activity of cells was assessed following the procedure described in 2.3.2.

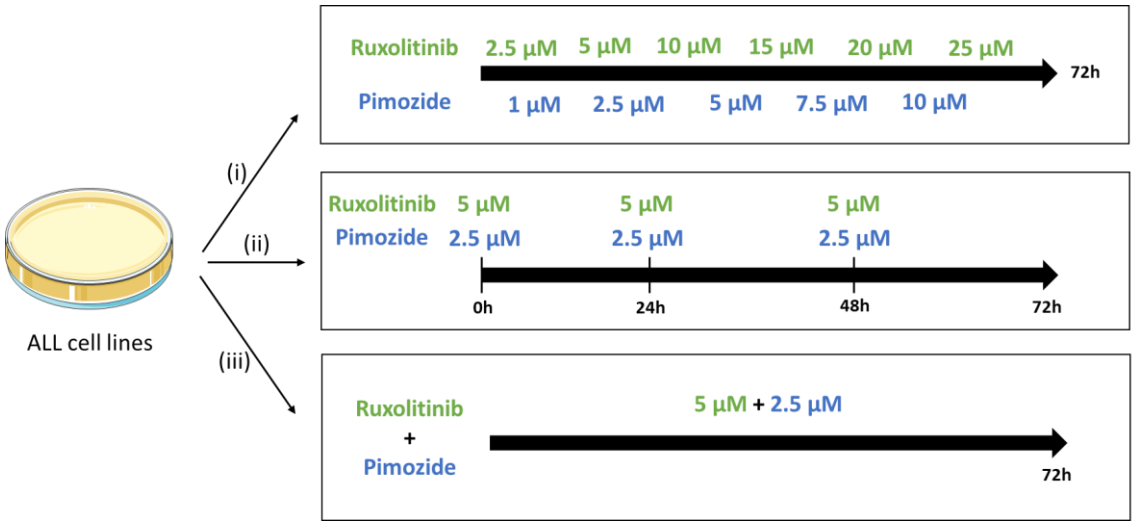

Figure 19 – Therapeutic schemes tested. (i) Single administration with increasing concentrations of Ruxolitinib (from 2.5 µM to 25 µM) and Pimozide (from 1 µM to 10 µM) for a period of 72 hours; **(ii)** Daily administration of Ruxolitinib (5 µM) and Pimozide (2.5 µM) in monotherapy at 24 and 48 hours; **(iii)** Combined administration of both inhibitors: 5 µM of Ruxolitinib and 2.5 µM of Pimozide.

2.3.2 Evaluation of metabolic activity of cells by Resazurin Assay

In order to evaluate the possible cytotoxic and cytostatic effects of Ruxolitinib and Pimozide in ALL cell lines, metabolic activity of these cells was determined through resazurin assay.

Resazurin is a dark blue and non-fluorescent oxidized form that is reduced to resorufin which colours pink and is highly fluorescent (206). Metabolic active cells reduce resazurin to resorufin since this dye is an intermediate electron acceptor in the mitochondrial electron transport chain. Thus, the amount of resorufin produced is proportional to the number of metabolic active cells, which represents the number of viable cells.

The 697, CEM, JURKAT and MOLT4 cell lines were incubated at an initial density of 0.3 x 10^6 cells/mL and REH at 0.5 x 10^6 cells/mL, in a 96-well plate for 72 hours. At each 24 hours of incubation, resazurin (Sigma-Aldrich, USA) was added to the cells. Using a spectrophotometer (Synergy ™ HT Multi-Mode Microplate Reader, BioTek Instruments, USA), absorbance at 570 nm (reduced form, i.e., resorufin) and 600 nm (oxidized form, i.e., resazurin) was read and relative metabolic activity of cells was assessed (**Equation 5**).

$$Metabolic\ activity\ (\%) = \frac{A_{(570-600)}\ sample - A_{(570-600)} blank}{A_{(570-600)} control - A_{(570-600)}\ blank} \times 100 \qquad \textbf{(Equation 5)}$$

The results represent mean ± SEM of 5 independent assays.

2.3.3 Cell death assessment by Flow cytometry

Flow cytometry is based on measurement of optical and fluorescence characteristics of cells when they pass across a light source (207). Scattering of light can be used to measure size (forward scatter) and morphological complexity (side scatter) of cells.

Apoptosis is defined by structural changes in cells (208). Morphological changes such as compaction and segregation of chromatin, condensation of the cytoplasm and plasma membrane alterations are the earliest events in apoptosis. Alterations in the plasma membrane include the translocation of phosphatidylserine (PS) from the inner leaflet to the outer. PS is a phospholipid negatively charged that is found in the inner leaflet of live cells. In presence of calcium, annexin V has high affinity to phospholipid with negative charge and binds to it allowing the determination of cells in apoptosis (209).

The integrity of plasma membrane is compromised in late events of apoptosis and in necrosis. 7-Aminoactinomycin D (7-AAD) is a fluorescent compound with low liposolubility that binds to DNA and emits fluorescence. Cells in late apoptosis and necrosis that have disrupted plasma membranes allow the entrance of 7-AAD and its access to the DNA turning staining (210,211). The double staining of the cells with

annexin-V and 7-AAD turns possible to distinguish viable cells, cells in early apoptosis, necrotic cells and cells in late apoptosis or necrosis. While live cells (viable) are annexin-V and 7-AAD negative, cells in late apoptosis/necrosis are positive for both. Cells in early apoptosis are annexin-V positive but 7-AAD negative. On the other hand, necrotic cells are annexin-V negative but 7-AAD positive (209,211).

For this purpose, cell lines exposed and not exposed to JAK-STAT inhibitors were stained with annexin-V (BioLegend) and 7-AAD (BioLegend). After incubation for 72 hours, one million of cells of each treatment were collected and washed by centrifugation with phosphate-buffered saline (PBS) during 5 minutes at 1000 xg. Afterwards, the *pellet* was resuspended in 100 µL of annexin-binding buffer and incubated with 2.5 µL of annexin-V and 5 µL of 7-AAD during 15 minutes in dark. After incubation, 300 µL of annexin-binding buffer was added and cells were analysed by FACSCalibur (BD Bioscience, USA) flow cytometer. Data were obtained using CellQuest™ (BD Bioscience, USA) and the results were analysed by Paint-a-Gate™ (BD science, USA). The results are expressed in percentage of cells, of each subpopulation positive or negative for annexin-V and for 7-AAD and shown as mean ± SEM, of 5 independent assays.

2.3.4 Evaluation of cell death by optical microscopy

To assess the type of death induced by JAK-STAT inhibitors, morphologic aspects of the ALL cell lines exposed to drugs were analysed by optical microscopy. For that, $5x10^5$ cells were collected and centrifuged at 300 xg for 5 min. Afterwards, cells were washed with PBS and once again centrifuged. FBS was then added to washed cells for better adhesion to the slide and smears were made. The smears were stained with *May-Grünwald* (Sigma Aldrich, USA) solution for 5 min and washed with distilled water for 1 min. Then, slides were stained for 15 min with *Giemsa* (diluted 1:3 with distilled water). Finally, the stained smears in *May-Grünwald-Giemsa* were washed in water stream and air dried. Morphologic analysis was performed in the optic microscope Nikon Eclipse 80i, equipped with a digital camera DMX 1200 F, and images were acquired in programme NIS-Elements D.

2.3.5 Cell cycle assessment

Cell cycle progression analysis by flow cytometry is based on DNA content of the cells. Staining the cells with specific dyes that emit fluorescence such as propidium iodide can be used to quantify DNA in cells, since the emitted fluorescence is proportional to DNA amount present in cells. Propidium iodide (PI) is a fluorescent dye that binds DNA double-strands as requires blue light to emit fluorescence. Due the fact that PI binds also double-stranded RNA, the addition of RNase to the staining solution is needed (212–214). During the cell cycle, the amount of DNA in cells changes. Cells in G_0/G_1 phase have the same amount of DNA whereas cells in G_2/M have twice of the amount of DNA. The S phase has intermedium DNA amount since in this phase occurs DNA synthesis and is located between G_0/G_1 and G_2/M phases. Moreover, apoptotic cells population are represented by a sub G_0/G_1 peak since they have a lower quantity in DNA content due to DNA fragmentation characteristic of apoptosis (214).

In order to evaluate the anti-proliferative of JAK-STAT inhibitors, ALL cell lines were incubated in absence and presence of Ruxolitinib and Pimozide. After 72 hours of incubation, one million of cells were collected and washed with PBS by centrifuging 1000 xg for 5 minutes. After that, 200 µL of ethanol 70 % was added and cells were incubated during 45 minutes at 4º C. Then cells were washed with PBS and centrifuged 1000 xg for 5 minutes. Finally, 300 µL of PI/RNase (Immunostep, Spain) was added. The DNA content was analysed in the flow cytometer FACSCalibur (BD Bioscience, USA), using ModFit LT™ software. The results are the mean ± SEM of 5 independent assays expressed in percentage (%) of cells in each phase of cell cycle.

2.4 Statistical analysis

Statistical analysis of all the data collected was performed using GraphPad® Prism version 7.04, as well as graphics design. To determine the statistical significance of the differences between the experimental groups, the non-parametric test of Kruskal-Wallis, and the multiple comparisons test of Dunn´s, were made. Additionally, the half

maximal inhibitory concentration (IC_{50}) of ruxolitinib and pimozide was calculated through a nonlinear logistic regression, applied to the results at 72 hours of exposure. Results are present as mean ± SEM and differences between means of the groups were considered significant for p-values lower than 0.05 (*p-values ≤ 0.05 and **p-values ≤ 0.01).

Chapter 3 – Results

3.1 Characterization of ALL cell lines

In this work, five acute lymphoblastic leukemia cell lines were used for metabolic characterization, namely 697, REH, CEM, JURKAT and MOLT4 cells. The first two are models of B-ALL and the rest consist in T-ALL models. These cells were cultured in a nutrient medium and maintained at 37ºC in a humidified atmosphere with 5% of CO_2. The proliferation and cell viability of each cell line is shown in **Figure 20A** and **20B**, respectively.

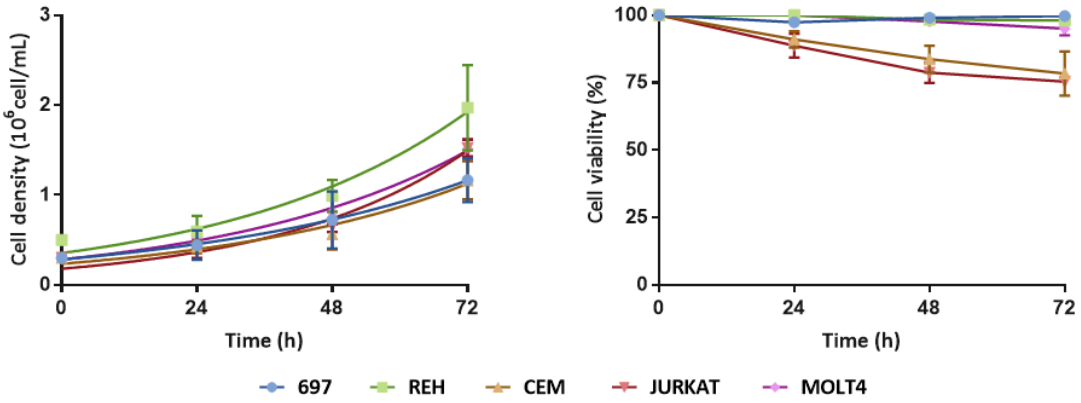

Figure 20 – Proliferation (A) and cell viability (B) of B-ALL cell lines (697 and REH), and T-ALL cell lines (CEM, JURKAT and MOLT4). Cell lines were culture at optimal growth conditions for 72 hours. The initial density of 697, CEM, JURKAT and MOLT4 cell lines was 0.3 x 10^6 cells/mL, and 0.5 x 10^6 cells/mL for REH. At each 24 hours, cell density and cell viability were evaluated, by trypan blue exclusion assay. The results are express the mean ± SEM of 3 independent assays.

All cell lines maintained an exponential growth throughout 72 hours of experiment (**Figure 20A**). JURKAT was the cell line that displayed the shortest doubling time and 697 cells the longest, approximately, 23 hours and 35 hours, respectively. REH and MOLT4 cells doubled their initial density after, approximately, 30 hours, while CEM required, approximately, 32 hours.

Regarding cell viability, all cell lines displayed a viability above 75% after 72 hours of proliferation (**Figure 20B**). REH, 697 and MOLT4 cell lines maintained the highest viability, standing above 95%, while JURKAT and CEM cells presented the lowest viability, of approximately, 75% and 78%, respectively.

The *CALR* gene was characterized in all cell lines, namely by the expression levels and the mutational status. The expression levels were assessed by qPCR and mutational

analysis of exon 9 of CALR gene was evaluated by Sanger sequencing. As shown in **Figure 21**, *CALR* expression levels were similar in 697, REH, CEM and MOLT4 cell lines. JURKAT cells exhibited higher expression of *CALR,* comparatively with the remaining cell lines. The differences of *CALR* expression levels were statistically significative comparing 697 and JURKAT cell lines (p=0.0349). In terms of mutations, no mutations on exon 9 of *CALR* gene were found in any of ALL cell lines in study.

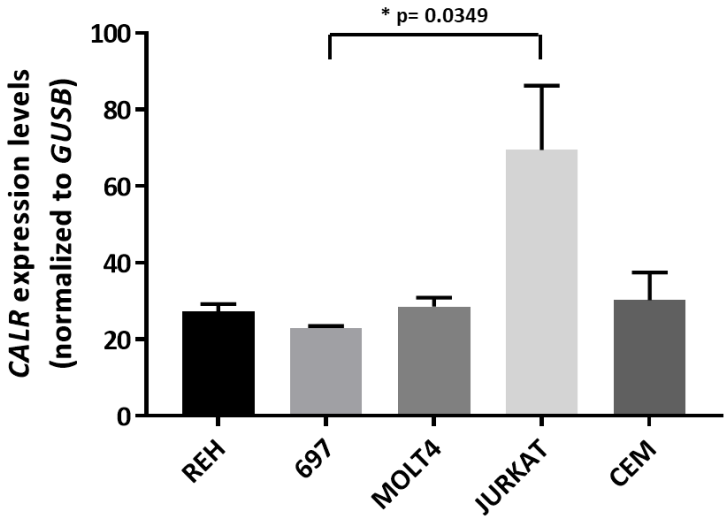

Figure 21 – Gene expression levels of *CALR* in ALL cell lines. The results were normalized to the reference gene *GUSB* and data was expressed as mean ± SEM of 3 independent assays. Statistical analysis was performed by Kruskal-Wallis test, followed by Dunn´s post-hoc test. *p<0.05

3.2 Evaluation of the therapeutic potential of Ruxolitinib and Pimozide

Ruxolitinib (RUX) and Pimozide (PIM) have been pointed as promising drugs to targeting JAK-STAT signalling pathway. Given this, we evaluated the therapeutic potential of RUX and PIM in B- and T-ALL in vitro cell models. Different schemes of administration were tested and resazurin assay was used to determine the metabolic activity of each cell line, as described in methods section.

3.2.1 Effect of Ruxolitinib on the metabolic activity in monotherapy

In **Figure 22** is shown the effect of single dose administration of increasing concentrations of RUX in ALL cell lines.

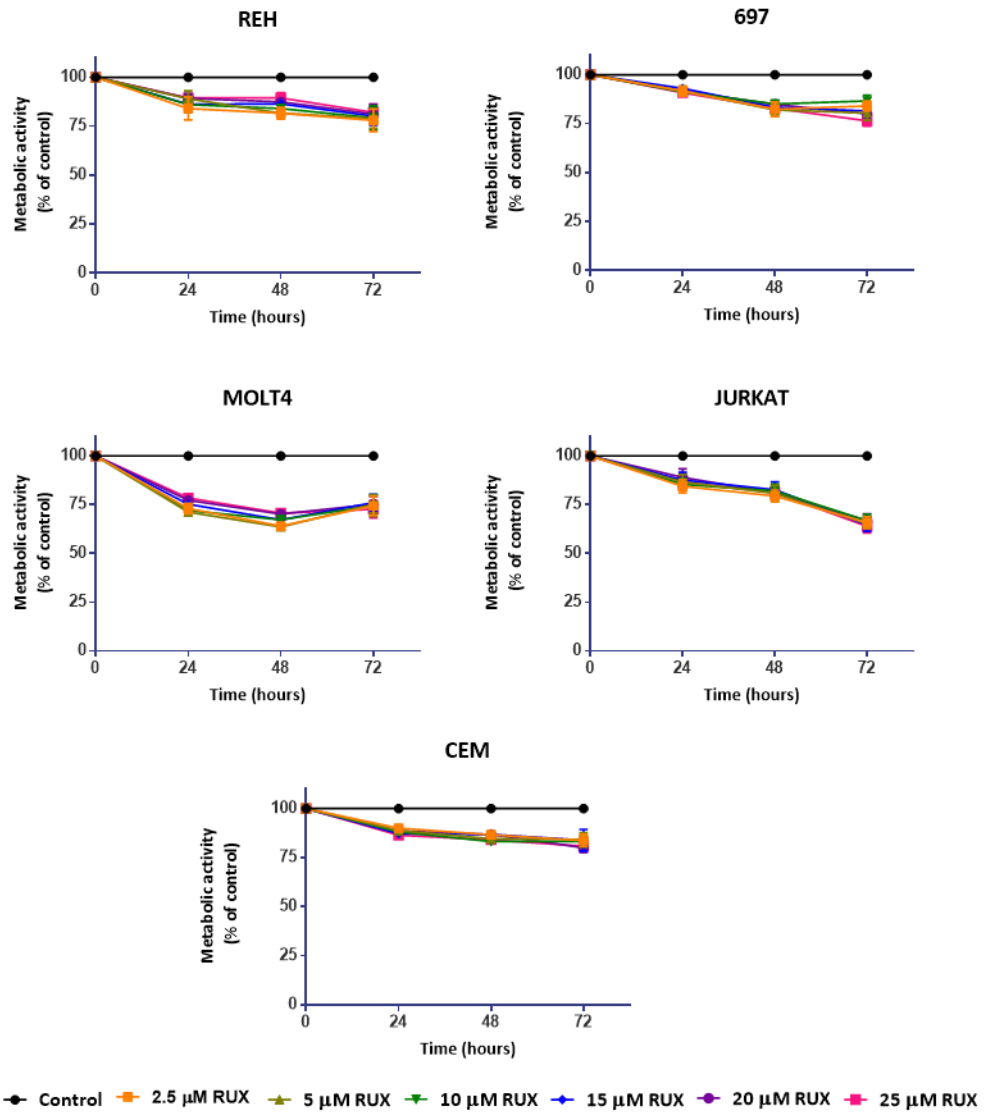

Figure 22 – Effect of Ruxolitinib (RUX) in monotherapy on metabolic activity of ALL cell lines. All cell lines were incubated at density of $0.3x10^6$ cells/mL except REH ($0.5x10^6$ cells/mL) in the absence (control) and in the presence of increasing concentrations of RUX, for 72 hours. The metabolic activity of cells was assessed at each 24 hours by the resazurin assay. The results are expressed in percentage (%) normalized to control. Data represent the mean ± (SEM) of 5 independent assays.

As seen in **Figure 22**, the effect of RUX seems to be time- and cell-dependent with no influence of dose. The highest reduction on metabolic activity occurred after 72 hours of incubation with this JAK inhibitor. The half maximal inhibitory concentration (IC_{50}), which corresponds to the concentration of drug needed to inhibit 50% of cell metabolism, was calculated mathematically for this time point. JURKAT cells seems to be the most sensitive model to RUX, with an IC_{50} of 26.45 µM, while CEM cell line was the less affected with an IC_{50} of 73.67 µM. Both REH and 697 cell lines presented an IC_{50} of 66.36 µM and 66.35 µM, respectively, and for MOLT4 cells the value was 44.85 µM.

3.2.2 Effect of daily administration of Ruxolitinib on the metabolic activity of ALL cell lines

It was also analysed if the method of administration has impact on the metabolic activity of the ALL cell lines.

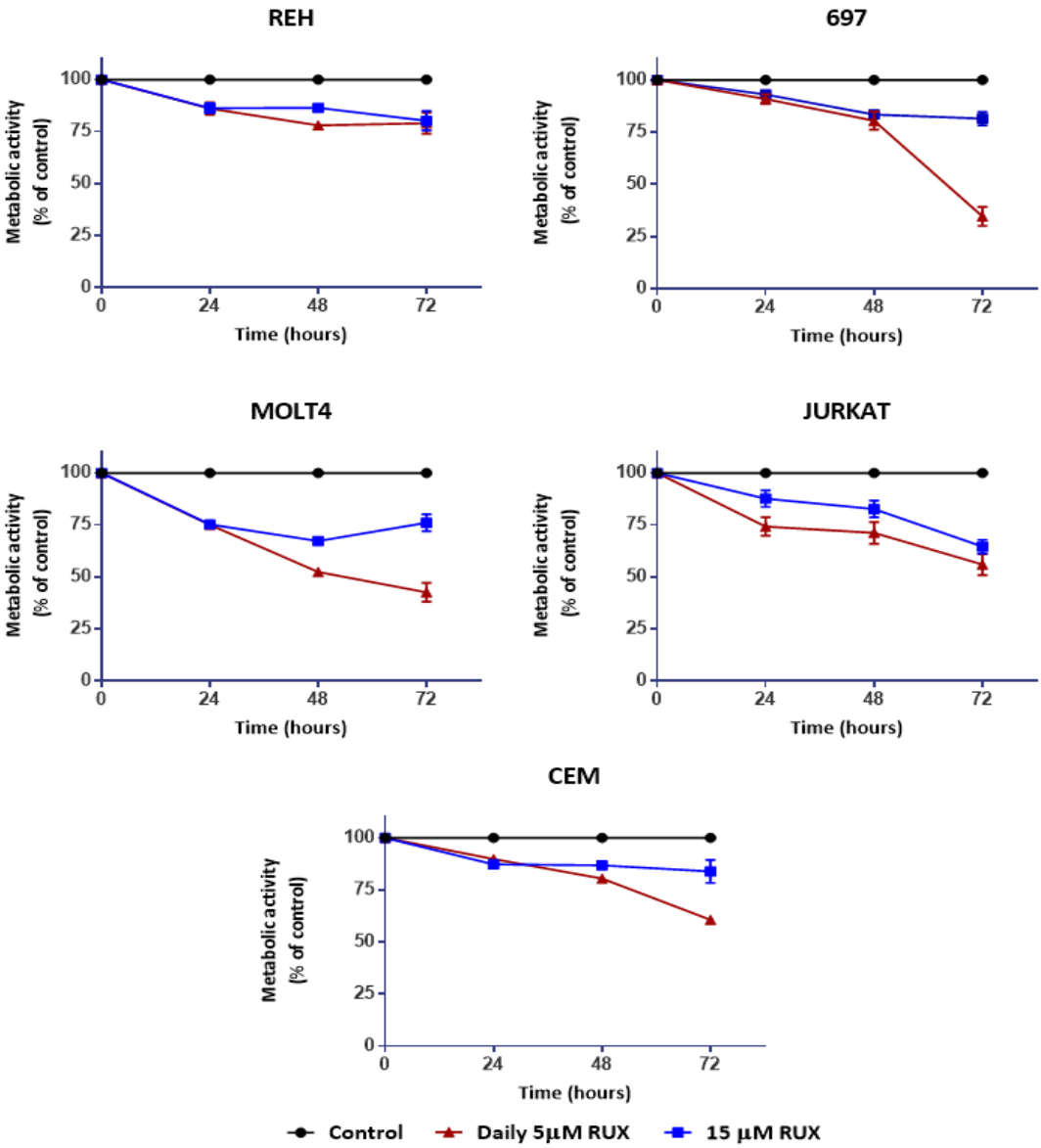

Figure 23 – Daily administration effect of RUX on metabolic activity of REH, 697, MOLT4, JURKAT and CEM cells. All cell lines were incubated at density of $0.3x10^6$ cells/mL except for REH cells ($0.5x10^6$ cells/mL), for 72 hours. RUX was administrated in single dose (15 µM) and in daily administration scheme (5µM every 24 hours). The metabolic activity of cells was assessed at each 24 hours by resazurin assay. The results are expressed in percentage (%) normalized to control. Data represent the of mean ± SEM of 5 independent assays.

In this way, a lower dose of RUX (5µM) was added to cell culture at each 24 hours, comparatively with 15µM single dose. The effect of ruxolitinib in REH, 697, MOLT4, JURKAT and CEM, following this method of administration is present in **Figure 23**.

The daily of ruxolitinib seems to have a different impact on the metabolic activity according to ALL cell lines (**Figure 23**). The 697 cells presented the highest results with daily administration, while in REH cell line no differences between schemes of administration were observed. After 72 hours of incubation, single administration of 15 µM of RUX induced a reduction of, approximately, 19% on metabolic activity of the 697 cell line. Comparatively, this reduction was exacerbated by daily administration of 5 µM RUX, causing a decrease of approximately 65% of the metabolic activity of these cells. MOLT4 was the second most sensitive cell line to this scheme of administration. At 72 hours, daily dose of RUX reduced approximately 58% of the metabolic activity of these cells, while single administration reduced only 24%. Moreover, daily administration of RUX inhibited approximately 40% of the metabolic activity of CEM cell line, whereas 15 µM in single administration inhibited approximately 17%. Similarly to REH cells, the effect obtained with daily administration of RUX in JURKAT cell line appears to be identical to single administration scheme.

3.2.3 Effect of Pimozide on the metabolic activity in monotherapy

STAT5 is a downstream effector of JAK2, being responsible for regulation of target genes involved in cell proliferation and survival. Thus, it was also tested the effect of inhibition of STAT5 by Pimozide (PIM) on the metabolic activity of ALL cells. The cell lines were incubated for 72 hours with increasing concentrations of PIM, which ranged between 1µM and 10µM.

As observed in **Figure 24**, the effect of PIM on the metabolic activity was time-, dose- and cell-dependent. Higher concentrations of PIM appear to induce higher reduction on metabolic activity in all cell lines and the effect of this inhibition was more visible after 72 hours. Therefore, mathematical IC_{50} was calculated for this time point.

MOLT4 appears to be the most sensitive cell line to PIM effect, having the lowest mathematical IC_{50} calculated of approximately 1.83 µM. On the other hand, REH seems to be less sensitive to PIM inhibition, which is translated to highest mathematical IC_{50} of approximately 10 µM. The IC_{50} for JURKAT cell line was of approximately 5.28 µM, while the IC_{50} of 697 and CEM cell lines was 9.03 µM and 9.18 µM, respectively.

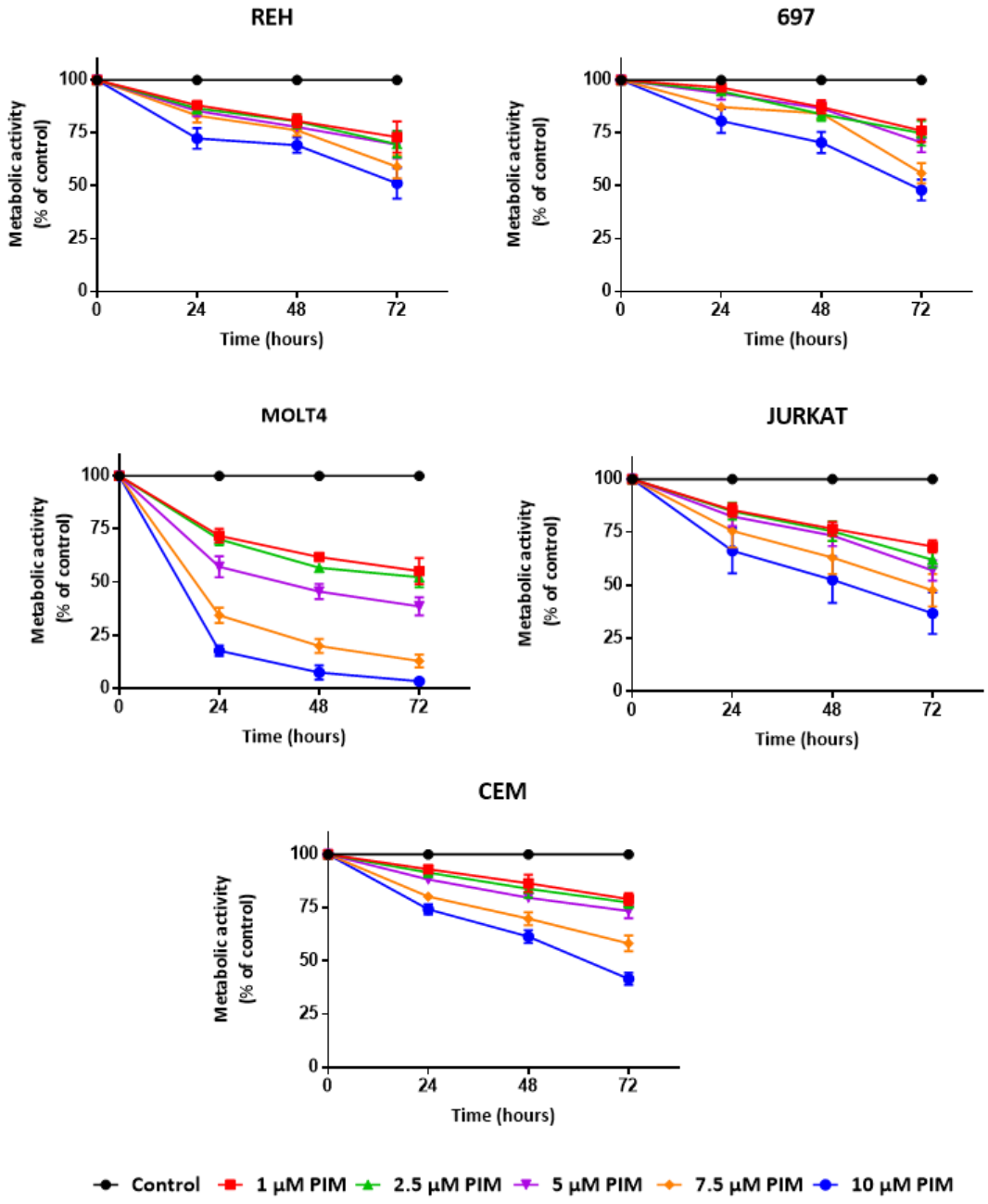

Figure 24 – Effect of Pimozide (PIM) in monotherapy on metabolic activity of ALL cell lines. All cell lines were incubated at density of $0.3x10^6$ cells/mL except for REH cells ($0.5x10^6$ cells/mL), in the absence (control) and in presence of increasing concentrations of PIM, for 72 hours. The metabolic activity of cells was assessed at each 24 hours by resazurin assay. The results are expressed in percentage (%) normalized to control. Data represent the mean ± SEM of 5 independent assays.

3.2.4 Effect of daily administration of Pimozide on the metabolic activity of ALL cell lines

The administration of lower doses of PIM every day may cause a greater inhibition on the metabolic activity of cell lines comparatively with single administration. For that, we evaluated daily administration of 2.5 µM of pimozide given every 24 hours comparing with 7.5 µM in single dose (**Figure 25**).

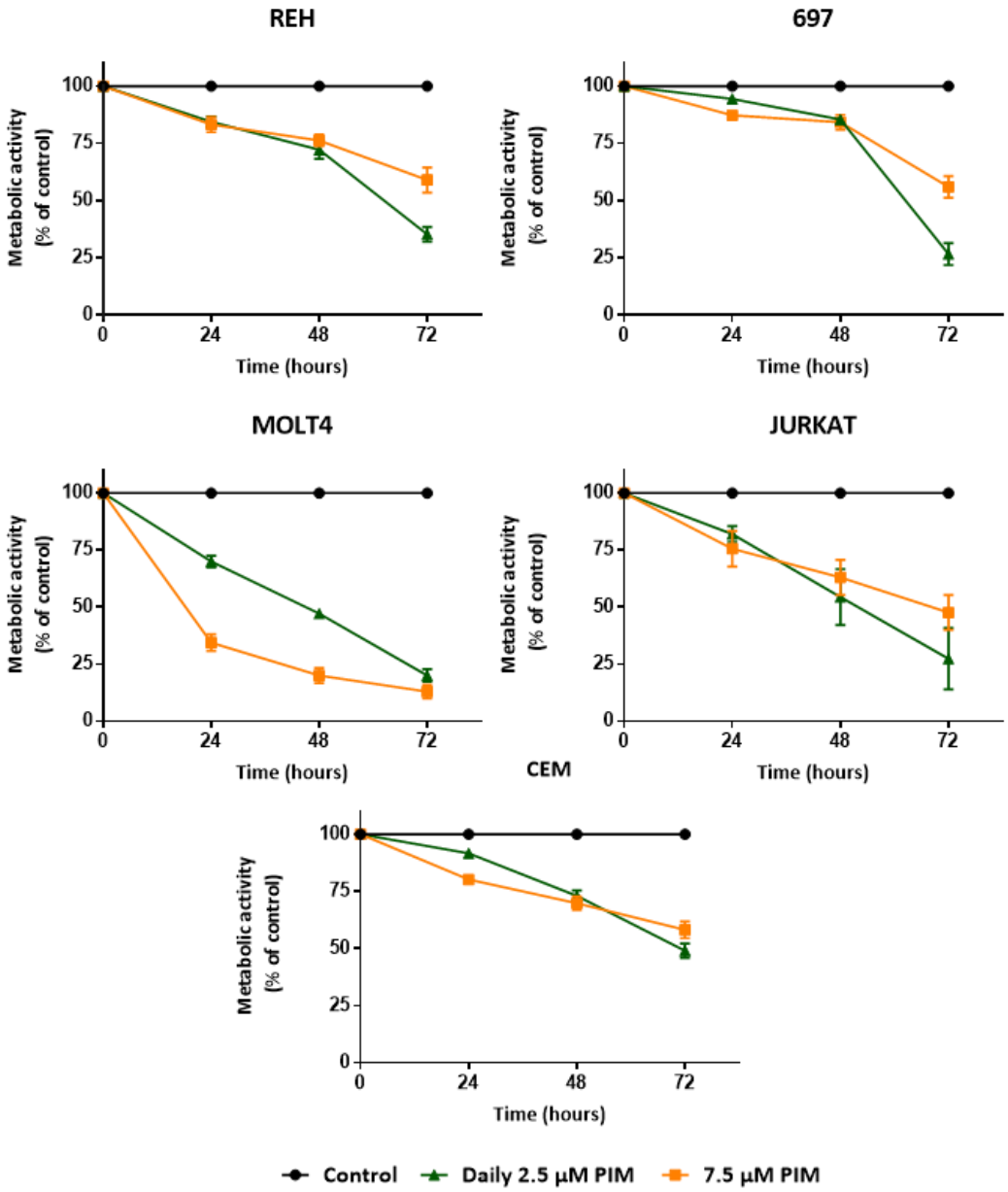

Figure 25 - Daily administration effect of PIM in ALL cell lines REH, 697, MOLT4, JURKAT and CEM. All cell lines were incubated at density of $0.3x10^6$ cells/mL except for REH cells ($0.5x10^6$ cells/mL), for 72 hours. PIM was administrated in single dose (7.5 µM) and in daily administration scheme (2.5µM every 24 hours). The metabolic activity of cells was assessed at each 24 hours by resazurin assay. The results are expressed in percentage (%) normalized to control. Data represent the mean ± SEM of 5 independent assays.

As shown in **Figure 25**, the way of administration of PIM seems to influence the metabolic activity of ALL cell lines differently. After 72 hours of incubation, this effect was more visible in B-ALL cell lines. For 697 and REH cells, the daily administration lead to a reduction of ≈73% and 65% in metabolic activity, comparatively with single administration that resulted in a decrease of 44% and 41%, respectively. Additionally, daily administration of PIM induces a decrease of 73 % in the metabolic activity of JURKAT cells, while single administration reduces the cell activity in 52%. CEM cell line results were less pronounced when comparing these two schemes of administration, with 51 % of metabolic inhibition in daily dose administration and 42% in single dose. MOLT4 cell line seem to be affected by PIM independently of the scheme of administration. Single administration of PIM inhibits 87% of the metabolic activity of these cells, and daily administration induces a reduction in 80% of the metabolic activity.

3.2.5 Evaluation of combined administration of Ruxolitinib and Pimozide on the metabolic activity

After evaluating the effect of ruxolitinib and pimozide in monotherapy we analyse if the combination of both JAK-STAT inhibitors may result in potentiation of effect comparing to single administration schemes. To do that, ALL cell lines were incubated simultaneously with lower concentrations of both inhibitors, 5 µM of RUX and 2.5 µM of PIM (**Figure 26**).

The combined administration of both RUX and PIM did not result in additive or synergistic effect in B- and T-ALL models. In all cell lines, the effect of drugs in combination were very similar to the ones obtained with PIM in single administration. Even so, MOLT4 appears to be the most affected cell line with the association of both drugs. After 72 hours of incubation, the association of both inhibitors induces a reduction in about 60% of the metabolic activity of these cells, while RUX and PIM alone, induces a reduction of approximately 25 % and 48 %, respectively.

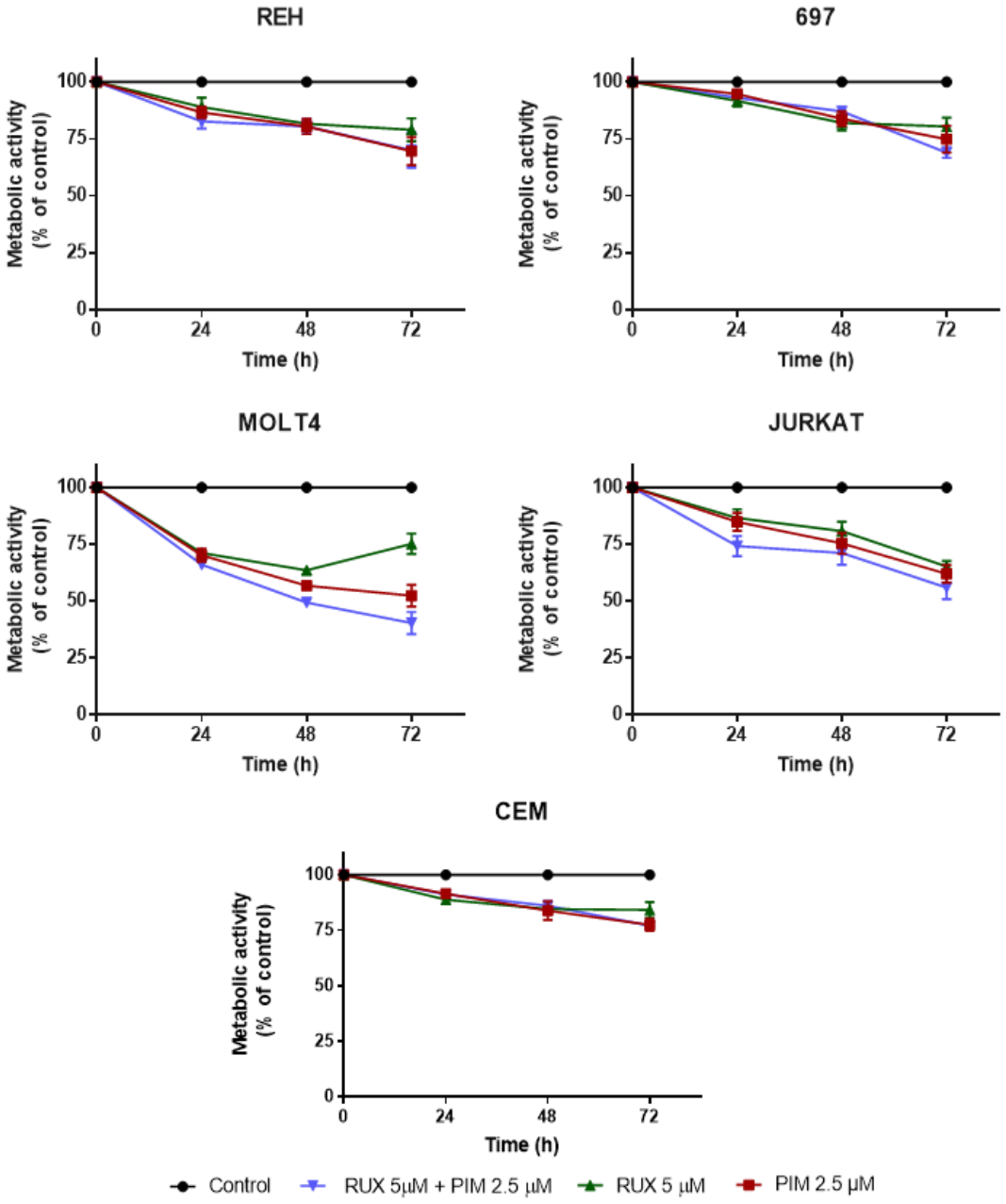

Figure 26 - Combined administration effect of RUX and PIM, in ALL cell lines. All cell lines were incubated at density of $0.3x10^6$ cells/mL except for REH ($0.5x10^6$ cells/mL), for 72 hours. In the combination scheme was used 5 µM of RUX plus 2.5 µM of PIM. The metabolic activity of cells was assessed at each 24 hours by resazurin assay. The results are expressed in percentage (%) normalized to control. Data represent the mean ± SEM of 5 independent assays.

3.2.6 Evaluation of the cytotoxic effect of Ruxolitinib and Pimozide by flow cytometry

To access if the both inhibitors in study present a cytotoxic effect, and if so, which type of cell death was preferential induced, flow cytometry studies were performed. The type of cell death was determined by flow cytometry, using the double staining of annexin-V and 7-AAD. For this purpose, each cell line was incubated with each inhibitor for 72 hours and the results were shown in **Figure 27.**

As observed in **Figure 27**, both inhibitors induced a cytotoxic effect in all cell lines after 72 hours of exposure. PIM presented a greater cytotoxic effect in all cell lines than RUX, being mainly due to apoptosis activation with an increase of cells in initial apoptosis as well as in late apoptosis/necrosis.

The incubation of REH cell line with both inhibitors induces a decrease of viable cells, from 92% to 80% for RUX and to 51% for PIM (p=0.0034). This was accompanied with an increase of cells in initial apoptosis (2% increase for RUX; 22% for PIM p=0.0042) and late apoptosis/necrosis (7% increase for RUX; 14% for PIM p=0.0032). The most affected cell line by both inhibitors was the 697 cell line. In this model, it was observed a decrease in 18% and 67% (p=0.0012) of viable cells exposed to RUX and PIM, respectively. The percentage of cells in initial apoptosis (increase of 15% for RUX and of 49% for PIM p=0.0012), late apoptosis/necrosis (increase of 2% for RUX and of 10% for PIM p=0.0031) and necrosis (increase of 1 % for RUX and of 8 % for PIM, p=0.0086), increased when exposed to RUX and PIM. In MOLT4, live cells percentage decreased 3% and 13% when exposed to RUX and PIM (p=0.0014), respectively. Moreover, there was a slightly increase of cells in initial apoptosis (increase of 1% for RUX and of 4% for PIM), late apoptosis/necrosis (increase of 1 % for RUX and of 4 % for PIM) and necrosis (increase of 1 % for RUX and of 4 % for PIM). In JURKAT cell line, 72 hours of incubation with RUX and PIM promoted a reduction in the percentage of live cells, from 84% to 79% and 38% (p=0.0102), respectively. However, it was observed an increase of cells in initial apoptosis (increase of 1% for RUX and of 24% for PIM p=0.0219), late apoptosis/necrosis (increase of 4% for RUX and of 11% for PIM p=0.0136) and necrosis only in cells treated with PIM (increase of 6% for PIM, p=0.0297). Lastly and in concordance with other cell lines, it was observed also a reduction of live CEM cells exposed to JAK-STAT inhibitors

(decrease of 5% for RUX and of 35% for PIM p=0.0059). The percentage of these cells in initial apoptosis (increase of 3% for RUX and of 25% for PIM p=0.0068), late apoptosis/necrosis (increase of 3% for RUX and of 9% for PIM p=0.0034) and necrosis (increase of 1% for PIM).

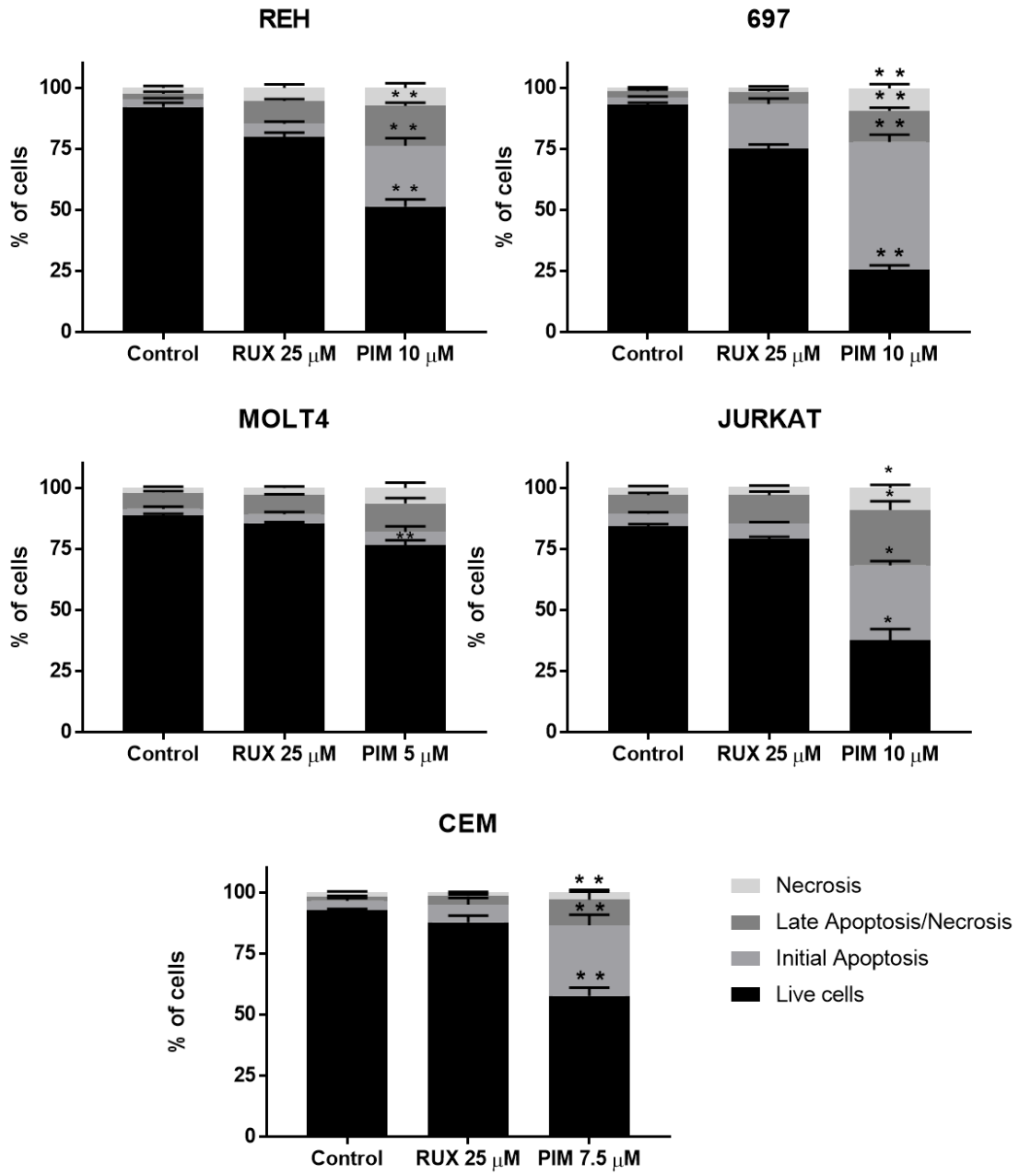

Figure 27 – Cytotoxic effect of Ruxolitinib and Pimozide in ALL cell lines. All these cells were incubated at density of $0.3x10^6$ cells/mL except REH cells ($0.5x10^6$ cells/mL), with concentrations of RUX and PIM indicated in figure, for 72 hours. After the period of incubation, cells were marked with annexin-V and 7-AAD and cell death was determined by flow cytometry. Data were expressed in percentage (%) and represent mean ± SEM of 3 to 5 independent assays. Statistical analysis was performed by Kruskal-Wallis test, followed by Dunn´s post-hoc test. *p<0.05, **p<0.01.

3.2.7 Evaluation of the type of cell death induced by Ruxolitinib and Pimozide by optical microscopy

To confirm the results obtained by flow cytometry regarding the type of cell death induced by RUX and PIM, cell morphology was evaluated by optic microscopy using *May-Grünwald-Giemsa* staining (**Figure 28**). The results given by flow cytometry and present in section 3.2.6 on **Figure 27**, reveal that RUX and PIM promote cell death largely by Initial Apoptosis and Late Apoptosis/Necrosis, although these results need to be confirmed by another technique such as optic microscopy. Apoptosis is characterized by morphological features that are specific of this type of cell death. Cells undergoing apoptosis have contraction of cytoplasm, chromatin condensation and nuclear fragmentation known as pyknosis and karyorrhexis, respectively. Additionally, in this type of cell death it occurs the formation of small vesicles called apoptotic bodies from plasma membrane blebbing with cellular content. Unlike apoptosis, necrosis is an uncontrolled type of cell death where the main characteristic is plasma membrane disruption and consequent release of cellular content to the extracellular space. The morphological evaluation of cell lines agreed with results obtained in flow cytometry.

In **Figure 28**, is presented the observations under optic microscopy of the morphological aspect of ALL cell lines. All cell lines show morphological changes that are characteristic of apoptosis, such as contraction of cytoplasm, nuclear fragmentation and blebbing when treated with ruxolitinib and pimozide. These cells also exhibit cytoplasmic vacuolization that could be an indicator of autophagy, which is a process of self-degradation important to remove damaged organelles.

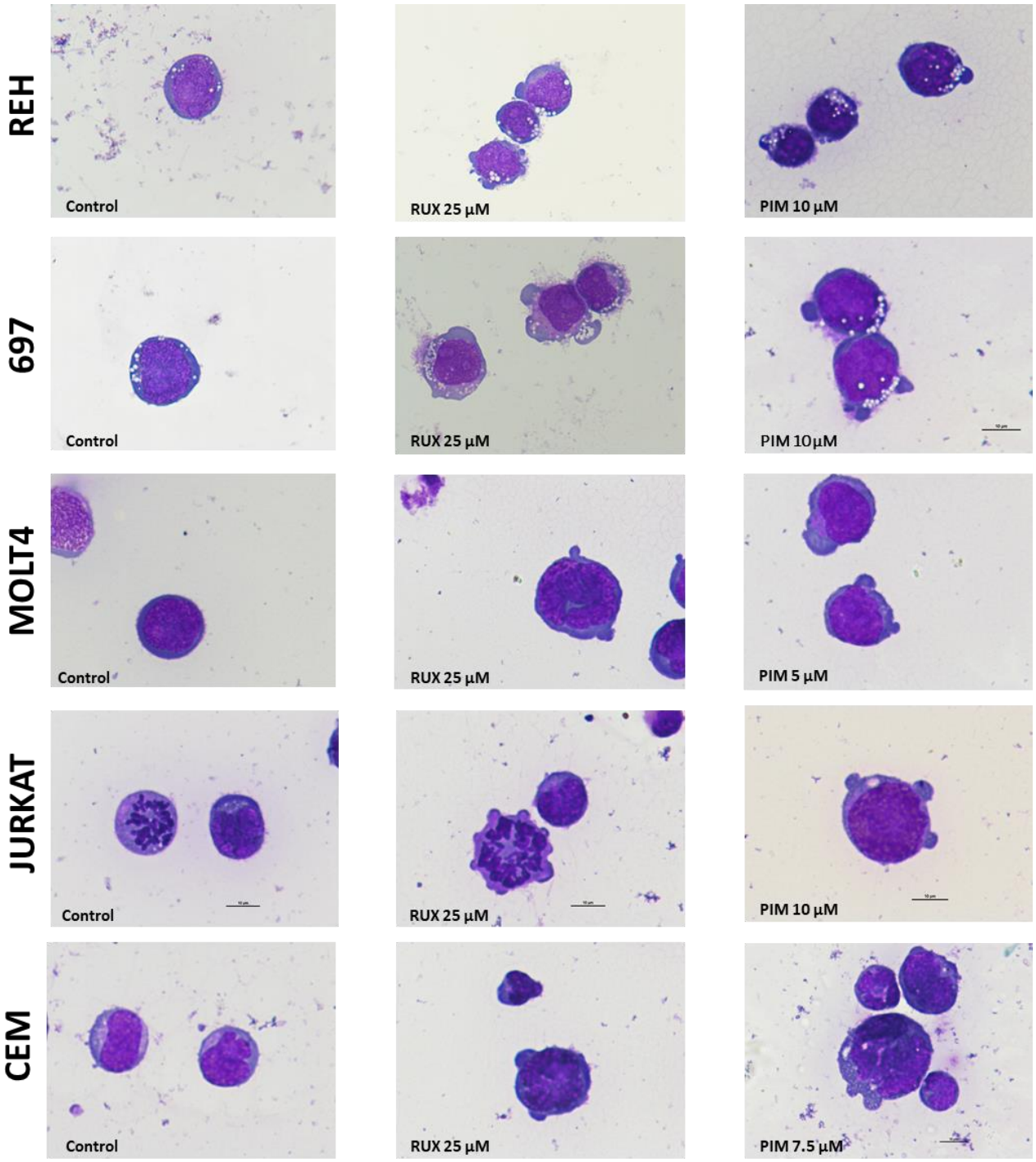

Figure 28 – Morphological analysis of ALL cell lines by optical microscopy. Cells were incubated for 72 hours in absence or presence of different concentrations of RUX and PIM, as indicated in the figure. The cells were stained with May-Grundwald-Giemsa and morphological characteristics evaluated by optic microscopy with 500x of magnification.

3.2.8 Evaluation of the cytostatic effect of Ruxolitinib and Pimozide in ALL cell lines

Additionally, Ruxolitinib and Pimozide may present a cytostatic effect leading to cell cycle arrest. For this purpose, the distribution of cells in each cell cycle phase was

evaluated by flow cytometry. ALL cell lines were incubated for a period of 72 hours in the absence and presence of RUX and PIM (**Table 3**).

Table 3 – Effect of RUX and PIM in cell cycle distribution in ALL cell lines.

		Sub G_1	G_0/G_1	S	G_2/M
REH	Control	0.0 ± 0.0%	58.7 ± 0.5%	32.0 ± 2.0%	9.3 ± 1.8%
	RUX 25 µM	1.0 ± 0.5%	59.0 ± 0.5%	33.0 ± 1.3%	8.0 ± 0.9%
	PIM 10 µM	**14.7 ± 1.6%***	63,3 ± 2.1%	18.0 ± 1.9%	4.0 ± 2.0%
697	Control	0.7 ± 0.5%	55.7 ± 0.3%	35.0 ± 0.5%	8.0 ± 0.5%
	RUX 25 µM	0.7 ± 0.3%	52.7 ± 1.0%	38.0 ± 0.9%	10.7 ± 1.0%
	PIM 10 µM	4.0 ± 0.9%	60.7 ± 3.8%	29.0 ± 1.7%	10.7 ± 2.0%
MOLT4	Control	2.0 ± 0.3%	39.6 ± 2.4%	53.0 ± 3.5%	7.4 ± 1.2%
	RUX 25 µM	2.2 ± 0.7%	39.4 ± 2.6%	52.8 ± 4.0%	8.0 ± 1.9%
	PIM 5 µM	2.6 ± 0.4%	39.2 ± 1.3%	52.6 ± 2.0%	8.2 ± 1.9%
JURKAT	Control	8.3 ± 1.5%	44.0 ± 0.9%	52.7 ± 0.7%	3.3 ± 0.7%
	RUX 25 µM	4.7 ± 1.2%	52.0 ± 0.9%	41.0 ± 0.5%	7.0 ± 0.5%
	PIM 10 µM	**30.3 ± 4.5%***	**61.3 ± 1.0%***	**31.7 ± 2.0%***	7.0 ± 1.3%
CEM	Control	0.8 ± 0.2%	36.8 ± 0.7%	41.8 ± 2.2%	21.5 ± 2.5%
	RUX 25 µM	0.8 ± 0.2%	35.5 ± 0.4%	40.5 ± 2.8%	24.0 ± 3.0%
	PIM 7.5 µM	3.3 ± 0.9%	38.8 ± 1.5%	37.0 ± 0.8%	24.3 ± 2.3%

All cell lines were incubated at density of $0.3x10^6$ cells/mL except for REH cells ($0.5x10^6$ cells/mL) with concentrations of RUX and PIM indicated in figure, for 72 hours. Data were expressed in percentage (%) and represent mean ± SEM of 5 independent assays. Statistical analysis was performed by Kruskal-Wallis test, followed by Dunn´s post-hoc test. *p<0.05.

As shown in **Table 3**, RUX did not induced a cytostatic effect in the five in vitro models used, while PIM induced a cell cycle arrest only in JURKAT cells. In this model was observed a significant increase of cells in G_0/G_1 phase, from 44% in control to 61.3% in PIM treatment (p=0.0219). This was followed a decrease of cells in S phase (52.7% in control to 31.7%, p=0.0219) and no alterations in G_2/M phase. Additionally, this analysis of DNA content allows the identification of apoptotic peak, that corresponds to cells

undergoing apoptosis. The exposure to PIM lead to an increase of apoptotic peak in all models, being the differences significant in REH and JURKAT cell lines. In the models, the percentage of cells identified in apoptotic peak increased to 14.7% ($p=0.0306$) and 30.3% ($p=0.0405$), respectively.

Chapter 4 – Discussion

ALL is characterized by abnormal proliferation of leukemic cells as a result of impaired maturation and differentiation processes (29). This is due to chromosomal aberrations and secondary genetic alterations which contribute for leukemic transformation of hematopoietic stem cells and committed lymphoid progenitors (37). Identification of these genetic lesions and determination of their clinical significance is important for diagnosis, risk stratification and development of targeted therapies (37). Therefore, ALL comprise multi-subtypes defined by structural abnormalities that differ in frequency and clinical outcomes (25,27).

Despite the fact that the majority of patients enter in remission after standard therapeutic regimens, a small portion of patients relapses (28). These subgroup of patients presents poor outcomes with conventional therapeutic regimens as a result of relapse-acquired genetic alterations that influence drug sensitivity of minor clones at diagnosis (37). Consequently, intensified cytotoxic chemotherapeutic schemes are administrated to these patients where about 47% of them achieve complete response (107,115,215). However, increasing toxicity of treatments is a clear limitation, especially in older patients with multiple relapses (28,215). Thus, it is crucial to develop therapies directed to specific molecular targets responsible for therapeutic failure.

The involvement of JAK-STAT pathway in leukemogenesis have been described, namely in ALL (79,81,122). This signalling pathway is important for regulation of many cellular processes involved in hematopoiesis, including cell growth, proliferation and differentiation (79). Genetic alterations that occurs in key components of this pathway, including *JAK2* and *STAT5* genes, promote dysregulation of JAK-STAT signalling and are linked to ALL pathogenesis (81,123,154). Constitutive activation of JAK-STAT cascade culminates in abnormal proliferation of leukemic cells (79). Additionally, the link between *CALR* mutations and JAK-STAT activation is well established in myeloproliferative neoplasms. Mutant calreticulin activates JAK2 and JAK-STAT signalling cascade through activation of MPL receptor (177,178). However, the role of *CALR* mutations in ALL is not known.

To understand the role of JAK-STAT signalling pathway, ALL cell lines were firstly characterized for *CALR* gene expression and *CALR* mutations, and subsequently the therapeutic potential of JAK-STAT inhibitors was evaluated.

Five genetically different *in vitro* cell models were chosen for this study and all of them represent a relapse model of ALL. B-ALL models, the 697 and REH cells, harbours the t(1;19) (q23;p13.3) encoding for *TCF3-PBX* and the fusion gene *ETV6-RUNX1* (*TEL-AML1*), respectively. In T-ALL models, the CEM cell line harbours the *NKX25-BCL11B* fusion gene, while JURKAT and MOLT4 cells are hypotetraploid and hypertetraploid, respectively.

Due to the relation between calreticulin and JAK-STAT signalling, the *CALR* expression levels and the mutational status of this gene were evaluated in the studied models. The results shown similar expression levels of *CALR* in 697, REH, CEM and MOLT4 cell lines, and higher expression in JURKAT cells. A previous study had identified that increased expression of *CALR* mRNA is associated with worse clinical prognosis in multiple malignancies including neuroblastoma, bladder cancer and mantle cell lymphoma (170). However, high expression levels of *CALR* in ALL and its clinical relevance is yet to be clarified. Additionally, ALL cell lines were screened for *CALR* mutations and no mutations in the exon 9 were found. Previous studies that investigated the mutation profile of *CALR* in ALL patients samples and in lymphoid cell lines also did not detected mutations in this gene, which is in accordance with our results (173,175). Despite these results, the number of patients involved in study and the type of population (exclusively Chinese) could be a limiting factor to the detection of *CALR* mutations (175). Therefore, additional studies are needed to explore the relevance of *CALR* expression and *CALR* mutations in ALL pathogenesis.

4.1. Therapeutic potential of JAK-STAT inhibitors

Dysregulation of JAK-STAT pathway occurs frequently in high-risk ALL cases, including Ph-like ALL, Down Syndrome-associated ALL, and T-ALL, being associated to poor prognosis and high-risk of relapse (49,122,126,149,216).

The JAK-STAT pathway is activated by cytokines, interleukins and growth factors which regulates cell proliferation, differentiation and survival (125). JAKs and STATs are key proteins responsible for signal transduction and integration by this signalling cascade. While JAKs are activated by their conjugated receptors, STATs are activated by JAKs, being responsible for regulation of the expression of genes involved in cell proliferation, differentiation and survival.

Genetic alterations that occur in key components of this pathway, including those in *JAK2* and *STAT5,* activate constitutively JAK-STAT, and are responsible for abnormal function of JAK-STAT signalling (86,216). Moreover, dysregulation of JAK2 and STAT5 activities are associated to chemotherapeutic and drug resistance that can lead to relapse (154,186). Based on this, targeting therapies, as the case of Ruxolitinib and Pimozide, has been developed to block JAK-STAT signalling pathways and promote cancer cells death.

RUX is a specific JAK2 inhibitor that have shown therapeutic efficacy in myeloproliferative neoplasms and in relapsed Ph-like ALL (124,184–186). Combination of RUX with chemotherapy has shown efficacy in Ph-like positive patients harbouring chromosomal rearrangements involving *JAK2* that failed induction therapy (186).

On the other hand, PIM is an inhibitor of STAT5, a downstream effector of JAK2 (184,193). PIM has shown cytotoxic and antiproliferative properties in CML cell lines that were resistant to tyrosine kinase inhibitors. This drug induced apoptosis and cell cycle arrest in CML cell lines. Additionally, PIM reduced STAT5 phosphorylation levels, decreasing the expression of STAT5 target genes involved in survival and growth of CML cells (193). In other MPN cell lines the same pattern of effect was observed, with an inhibition of *BCL-xl* and *MCL1* expression levels (195). While the therapeutic effect of PIM is well characterized in MPN, in ALL the potential effect remains unknown.

Therefore, the aim of this project was to evaluate the therapeutic potential of these two JAK-STAT inhibitors in vitro models of ALL and characterize the mechanisms of action. For that, the effect of these drugs was evaluated in monotherapy (single and daily administrations) and in combination in 697, REH, MOLT4, JURKAT and CEM cell lines.

First, it was evaluated the therapeutic potential of single doses administration of JAK2 inhibitor RUX on proliferation and viability of ALL cell lines. Our results indicate that single dose administration of RUX has a time- and cell dependent, but dose-independent effect. JURKAT and MOLT4 cells were the most sensitive cell lines to RUX, with and IC_{50} of 26.45 µM and 44.85 µM after 72 hours, respectively. In opposition, CEM cells were the less sensitive models to drug (IC_{50} of 73.67 µM at 72 hours). These results suggest that JURKAT and MOLT4 cell lines may be more dependent on JAK2 activation than REH, 697 and CEM cells. Previous study also reported a decrease in survival and proliferation

rate of JURKAT cells when expose to RUX, in accordance with our results (217). On the other hand, a second study with REH cell line reported that RUX had minimal or no effect on proliferation of this cell line (for concentrations between 0.025 μM and 0.4 μM) (218). In fact, our results indicate that REH cell line was less sensitive to RUX even for higher concentrations of RUX. This can be explained by the presence of *ETV6-RUNX1* fusion gene in REH cell line (198). The *ETV6-RUNX1* can promote increase cell survival and proliferation by binding to the erythropoietin receptor (EPOR) and activating not only JAK-STAT pathway, but also PI3K/AKT/mTOR pathway (219,220). In presence of ETV6-RUNX1, erythropoietin (EPO) activate JAK2 and AKT phosphorylation which induces STAT5 activation and transcription of pro-survival and antiapoptotic protein BCL-XL (220). The inhibition of JAK2 by RUX could prevent STAT5 activation and transcription of pro-survival genes. However, side activation of others signalling pathway such as PI3K/AKT/mTOR may function as a compensatory pathway, promoting cell proliferation and survival and contributing for maintenance of the leukemic state (221).

In 697, MOLT4 and CEM cell lines, it was observed a cell recovering between the 48 and 72 hours of RUX incubation, which may occur due to dissociation of RUX from JAK2 caused by the decreasing of RUX availability (222). Computing simulation of the mechanism of action of RUX suggests that the inhibition of JAK2 occurs through type I binding (223). In this type of binding, RUX targets the ATP-binding site of JAK2 in its active conformation, inactivating JAK2 kinase activity (223). Thus, RUX is an ATP mimetic that reversibly inhibits JAK2 but can dissociate from JAK2 once its availability decreases. This situation can be avoided changing the scheme of drug administration and could be improved by daily administration in order to obtain continuous inhibition (222).

Taken this into consideration, in the five *in vitro* models the effect of daily administration of RUX was evaluated. Our results show that daily administration of a lower dose of RUX has an intensified effect on the metabolic activity of all cell lines, except in REH cells, comparatively with single dose after 72 hours of incubation. The most sensitive cell line to this way administration was the 697 cell line, while in REH there were no differences between the two ways of administration. The 697 cell line harbours *TCF3-PBX1* (*E2A-PBX1*) fusion gene (196). A study reported that secondary mutations in JAK-STAT pathway were detected in B cell progenitors with *E2A-PBX1*

fusion gene, where cell lines and mouse E2A-PBX1 leukemia were sensitive to the presence of different concentrations of RUX, which is in accordance with our results (224). As stated before, side activation of additional pathways may justify the less sensitive of REH cells when exposed to the JAK2 inhibitor RUX (221).

In both MOLT4 and CEM cell lines, it is observed a slightly recuperation in the activity of these cells when exposed to single dose of RUX. However, with daily administration this was prevented, and a higher inhibition was observed in this scheme of administration.

Next, it was evaluated the therapeutic potential of single doses administration of STAT5 inhibitor PIM on proliferation and viability of ALL cell lines. Our results show that inhibition of STAT5 induced a reduction on the metabolic activity of ALL cell lines in a time-, dose- and cell-dependent manner. The MOLT4 is the most sensitive cell line to PIM effect (with an IC_{50} of 1.83 μM at 72 hours) while REH the less sensitive to STAT5 inhibition (with an IC_{50} of 10 μM at 72 hours). These results suggest that MOLT4 cells are more dependent on STAT5 activation to proliferate, while REH seems to be less dependent.

Besides its canonical functions where STAT5 is phosphorylated and activated by tyrosine kinases as JAK2, non-tyrosine phosphorylated STAT5 have additional functions in the nucleus, endoplasmic reticulum and Golgi apparatus which is less known (154). In fact, previous studies suggested that non-phosphorylated STAT5 can influence cell survival of leukemic cells (225). Since PIM inhibits STAT5 phosphorylation, non-phosphorylated STAT5 can acquire new functionalities and control the survival and proliferation of leukemic cells (193). This fact can explain why some cell lines are more resistant to STAT5 inhibition. On the other hand, this resistance can be justified by the fact that PIM inhibits STAT5 without inhibiting the kinase activity of JAK2, which can promote activation of parallel signalling pathways such as PI3K/AKT/mTOR that may sustain cell proliferation and survival (193).

In the next step was evaluated the effect of daily administration of PIM in ALL cell lines, to access the beneficial potential of this scheme of administration comparing to single dose administration. Therefore, our results suggest that daily administration of PIM have a higher inhibitory effect on the metabolic activity of REH, 697, JURKAT and

CEM cell lines. However, in MOLT4 cells both schemes of administration result in same effect after 72 hours of exposure being the most sensitive cell line to PIM effect.

The inhibition of JAK-STAT pathway at different two levels, by inhibition of JAK2 and STAT5, may provide a greater inhibitory effect in leukemia cells. Additionally, the use of lower concentrations of each drug can prevent the development of drug resistance and can reduce possible side-effects. Subsequently, the therapeutic effect of combination of both inhibitors, RUX and PIM, on proliferation and survival of the cell lines in study was evaluated. Previous studies have shown the therapeutic efficacy of dual inhibition of JAK2 and STAT5 in MPN cells (195). However, our results fail to show a synergistic effect between both drugs in ALL models. The results observed in combined treatment were very similar to the ones observed with PIM alone. This could be justified by the increased sensitivity of all models to the STAT inhibitor rather than to Ruxolitinib.

The inhibitory effect of JAK2 and STAT5 inhibitors observed on metabolic activity of our models can be mediated by two possible cellular events: cell death (cytotoxic effect) or cell cycle arrest (cytostatic effect).

To access the mechanism of cell death induced by both inhibitors in ALL cell lines, firstly cytotoxic effect of RUX and PIM was evaluated by flow cytometry and optical microscopy. Our results shown that both RUX and PIM inhibitors have a cytotoxic effect in ALL cell lines by inducing apoptosis. In all cell lines, occurred a decrease in the number of viable cells, followed by an increase of cells in initial apoptosis, late apoptosis/necrosis. The cytotoxic effect of both inhibitors is more evident in 697 cell line while MOLT4 cells were the less affected cell line. These results could be justified by a lower dose of PIM (5 µM) used in flow cytometry for MOLT4 cell line, comparing to 10 µM used for 697 and JURKAT cells. The cytotoxic effect induced by two inhibitors was confirmed by morphological evaluation of ALL cell lines exposed to RUX and PIM.

The morphological alterations observed in cell smears were characteristic of cell death by apoptosis and confirm the results obtained in flow cytometry analysis. Cytoplasmic projections (blebbing), cytoplasm contraction as well as nuclear contraction and fragmentation were some of the features observed in cell lines treated with RUX and PIM. Additionally, it was observed cell vacuolization which can be an indicator of autophagy. In fact, previous studies showed that RUX and PIM induced autophagy in the MPN cell line (SET2) and in mouse embryonic fibroblasts exposed to these inhibitors,

respectively (226,227). However, possible activation of autophagy in our cell lines should be confirmed with additional assays. For that, detection of activation levels of the mammalian target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK), which are major regulators of the autophagy process, should be performed (228).

Besides cytotoxic effect, RUX and PIM can also present a cytostatic effect on ALL cell lines and induce cell cycle arrest. Therefore, it was evaluated the cytostatic effect of RUX and PIM in ALL cell lines, by flow cytometry. Our results demonstrate that RUX did not present any cytostatic effect in the five models of ALL in study. On the other, PIM revealed a cytostatic effect only on JURKAT cells, inducing a cell cycle arrest in G_0/G_1 phase. Additionally, with the same technique was possible to observe an apoptotic peak in cells exposed to PIM. This data supports the activation of apoptosis in these cells, as a mechanism of cell death.

In line with our study, a previous study demonstrated that RUX in monotherapy had no significant cytotoxic effect in Ph-chromosome leukemia-propagating cells models (229). However, combination of RUX with nilotinib significantly induced apoptosis in those models. Regarding PIM, our results are in accordance with several reports in literature. It was reported that in AML, activation of STAT5 is essential for leukemogenesis, and inhibition of STAT5 by PIM induces cell death by apoptosis (230). Moreover, it was also reported that PIM induces cell cycle arrest and cell death by apoptosis in CML cell lines (193). Additionally, PIM induces the same cytotoxic effect in other MPN cells (195).

Overall, the results obtained come to elucidate the importance of JAK2-STAT5 signalling in cell proliferation and survival of ALL cell lines, and as a therapeutic target to treat ALL.

Chapter 5 – Conclusion and further perspectives

The JAK-STAT pathway is implicated in hematopoiesis and many other physiological processes, including those involved in cell differentiation, proliferation and survival. This pathway is an important signalling cascade responsible for the transduction of extracellular signals to the nucleus. Dysregulation and alterations in the main components of this pathway, particularly in JAK2 and STAT5, have been associated to ALL leukemogenesis. Thus, it is crucial the development of more targeted therapeutic approaches for these signalling components.

In conclusion, the results obtained in this study shown that Ruxolitinib (RUX) and Pimozide (PIM) can be potential therapeutic approaches in ALL models. The main observations of this work were:

- The ALL *in vitro* models used in the study did not present mutations on CALR gene and the expression levels were very similar across the models, excepted for JURKAT cells (highest expression detected).

- The inhibition of JAK2 by RUX, was time- and cell line-dependent, but dose-independent when in single administration. JURKAT were the most sensitive cells and CEM the most resistant cell line to this scheme of administration. Moreover, daily administration of RUX induced a reduction on metabolic activity that was cell line dependent. For this type of administration, 697 cell lines was the most sensitive in opposition to REH cells where no differences were observed according to the strategy of administration.

- The inhibition of STAT5 by single doses of PIM, was time-, dose- and cell line-dependent. MOLT4 was the most sensitive cell line to STAT5 inhibition and REH the most resistant. Moreover, daily administration of PIM was beneficial, especially in B-ALL models. 697 and REH cell lines were the most sensitive to daily inhibition of STAT5, while in MOLT4 no increasing of effect was observed.

- The combination of both inhibitors, RUX and PIM, in our models did not result in an additive or synergistic effect.

- Mechanistically, JAK-STAT inhibition by RUX and PIM induced had a cytotoxic effect in all cell lines, mediated by apoptosis induction. In addition, PIM demonstrated a cytostatic effect in JURKAT cell line, inducing a cell cycle arrest at G_0/G_1 phase.

Despite some limitations of this work, additional experiments are necessary to consolidate the previous results. Moreover, it would be interesting to characterize these cell lines for JAK2 and STAT5 mutations that are found in acute leukemias. Since there are evidence of side activation of other pathways, it would be interesting to test different associations of RUX and PIM with additional inhibitors targeting for example PI3K/AKT/mTOR pathway.

Chapter 6 – References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. Anticancer Res. 2018;33(6):2597–604.

2. Visvader JE, Lindeman GJ. Perspective Cancer Stem Cells: Current Status and Evolving Complexities. STEM. 2012;10(6):717–28.

3. Kreso A, Dick JE. Evolution of the Cancer Stem Cell Model. Stem Cell. 2014;14(3):275–91.

4. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell. 2011;144(5):646–74.

5. Landau DA, Carter SL, Getz G, Wu CJ. Clonal evolution in hematologic malignancies and therapeutic implications. Leukemia. 2014;28(1):34–43.

6. Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. Nature. 2013;501(7467):328–37.

7. Nowell PC. The clonal evolution of tumor cell populations. Science (80-). 1976;194(4260):23–8.

8. Greaves M, Maley CC. Clonal evolution in cancer. Nature. 2012; 481, 306-313.

9. Rieger MA, Schroeder T. Hematopoiesis. Cold Spring Harb Perspect Biol. 2012; 4(12)

10. Jagannathan-Bogdan M, Zon LI. Hematopoiesis. Dev. 2013; 140: 2463-2467

11. Orkin SH, Zon LI. Hematopoiesis: An Evolving Paradigm for Stem Cell Biology. Cell. 2008; 132(4):631-644

12. Orkin SH. Diversification of haematopoietic stem cells to specific lineages. Nature Reviews Genetics. 2000; 1:57-64

13. Doulatov S, Notta F, Laurenti E, Dick JE. Hematopoiesis: A human perspective. Cell Stem Cell. 2012;10(2):120–36.

14. Neumüller RA, Knoblich JA. Dividing cellular asymmetry: Asymmetric cell division and its implications for stem cells and cancer. Genes and Development. 2009;23(23):2675-2699

15. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, et al. A Bivalent Chromatin Structure Marks Key Developmental Genes in Embryonic Stem Cells. Cell. 2006;125(2):315-26

16. Boulais PE, Frenette PS. Making sense of hematopoietic stem cell niches. Blood. 2015;125(17):2621–9.

17. Schepers K, Campbell TB, Passegué E. Normal and leukemic stem cell niches: Insights and therapeutic opportunities. Cell Stem Cell. 2015;16(3):254-267

18. Passegue E, Jamieson CHM, Ailles LE, Weissman IL. Normal and leukemic hematopoiesis: Are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? Proc Natl Acad Sci . 2003;100(Supplement 1):11842–9.

19. Kondo M. Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. Immunol Rev. 2010;238(1):37–46.

20. Chen J, Odenike O, Rowley JD. Leukaemogenesis: More than mutant genes. Nature Reviews Cancer. 2010;10(1):23-36

21. Bernt KM, Armstrong SA. Leukemia Stem Cells and Human Acute Lymphoblastic Leukemia. Semin Hematol. 2009;46(1):33–8.

22. Lane SW, Gilliland DG. Leukemia stem cells. Semin Cancer Biol. 2010;20(2):71–6.

23. Kourti M, Vavatsi N, Gombakis N, Tzimagiorgis G, Sidi V, Koliouskas D, et al. Increased expression of multidrug resistance gene (MDR1) at relapse in a child with acute lymphoblastic leukemia. Pediatr Hematol Oncol. 2006;23(6):489–94.

24. Oldenborg P-A. CD47: A Cell Surface Glycoprotein Which Regulates Multiple Functions of Hematopoietic Cells in Health and Disease. ISRN Hematol. 2013;2013:614619.

25. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391-2405.

26. Taylor J, Xiao W, Abdel-Wahab O. Diagnosis and classification of hematologic malignancies on the basis of genetics. Blood. 2017;130(4):410–23.

27. Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, et al. The 2016 revision of the World Health Organization classi fi cation of lymphoid neoplasms. Blood. 2016;127(20):2375–91.

28. Terwilliger T, Abdul-Hay M. Acute lymphoblastic leukemia: a comprehensive review and 2017 update. Blood Cancer J. 2017;7(6):e577.

29. Inaba H, Greaves M, Mullighan CG. Acute Lymphoblastic Leukemia. NIH Public Access. 2013;381(9881):1–27.

30. Hunger SP, Mullighan CG. Acute Lymphoblastic Leukemia in Children. N Engl J Med. 2015;373(16):1541–52.

31. Biondi A, Scrideli CA, Cazzaniga G. Acute lymphoblastic leukemia. Mol Pathol Clin Pract Ed. 2016;116(5):561–77.

32. Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. The Lancet. 2008;371(9617):1030-1043.

33. Lee P, Bhansali R, Izraeli S, Hijiya N, Crispino JD. The biology, pathogenesis and clinical aspects of acute lymphoblastic leukemia in children with Down syndrome. Leukemia. 2016;30(9):1816-1823.

34. Sehgal S, Mujtaba S, Gupta D, Aggarwal R, Marwaha RK. High incidence of Epstein Barr virus infection in childhood acute lymphocytic lukemia: A preliminary study. Indian J Pathol Microbiol. 2010;53(1):63-67.

35. Gérinière L, Bastion Y, Dumontet C, Salles G, Espinouse D, Coiffier B. Heterogeneity of acute lymphoblastic leukemia in HIV-seropositive patients. Ann Oncol. 1994;5(5):437-440.

36. Ching-hon P, V RM, R DJ. Acute Lymphoblastic Leukemia. 2004;1535–48.

37. Iacobucci I, Mullighan CG. Genetic basis of acute lymphoblastic leukemia. Journal of Clinical Oncology. 2017;35(9):975-983

38. Moorman A V. New and emerging prognostic and predictive genetic biomarkers in B-cell precursor acute lymphoblastic leukemia. Haematologica. 2016;101(4):407–16.

39. Zhang X, Rastogi P, Shah B, Zhang L. B lymphoblastic leukemia/lymphoma: New insights into genetics, molecular aberrations, subclassification and targeted therapy. Oncotarget. 2017;8(39):66728–41.

40. Hunger SP, Lu X, Devidas M, Camitta BM, Gaynon PS, Winick NJ, et al. Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: A report from the children's oncology group. J Clin Oncol. 2012;30(14):1663-1669.

41. Mullighan CG. Molecular genetics of B-precursor acute lymphoblastic leukemia. Journal of Clinical Investigation. 2012. 122(10):3407–3415.

42. Barber KE, Harrison CJ, Broadfield ZJ, Stewart ARM, Wright SL, Martineau M, et al. Molecular cytogenetic characterization of TCF3 (E2A)/19p13.3 rearrangements in B-cell precursor acute lymphoblastic leukemia. Genes Chromosom Cancer. 2007;46(5):478-486.

43. Rambaldi A, Attuati V, Bassan R, Neonato MG, Viero P, Battista R, et al. Molecular Diagnosis and Clinical Relevance of t(9;22), t(4;ll) and t(l;19) Chromosome Abnormalities in a Consecutive Group of 141 Adult Patients with Acute Lymphoblastic Leukemia. Leuk Lymphoma. 1996;21(5-6):457-466.

44. Andersson AK, Ma J, Wang J, Chen X, Gedman AL, Dang J, et al. The landscape of somatic mutations in infant MLL-rearranged acute lymphoblastic leukemias. Nat Genet. 2015;47(4):330-337.

45. Holmfeldt L, Wei L, Diaz-Flores E, Walsh M, Zhang J, Ding L, et al. The genomic landscape of hypodiploid acute lymphoblastic leukemia. Nat Genet. 2013;45(3):242-252.

46. Safavi S, Paulsson K. Near-haploid and low-hypodiploid acute lymphoblastic leukemia: Two distinct subtypes with consistently poor prognosis. Blood. 2017;129(4):420-423.

47. Den Boer ML, van Slegtenhorst M, De Menezes RX, Cheok MH, Buijs-Gladdines JG, Peters ST, et al. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. Lancet Oncol. 2009;10(2):125-134

48. Roberts KG, Gu Z, Payne-Turner D, McCastlain K, Harvey RC, Chen IM, et al. High Frequency and Poor Outcome of Philadelphia Chromosome-Like Acute Lymphoblastic Leukemia in Adults. J Clin Oncol. 2017;35(4):394-401.

49. Roberts KG, Li Y, Payne-Turner D, Harvey RC, Yang YL, Pei D, et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. N Engl J Med. 2014;371(11):1005-1015.

50. Buitenkamp TD, Pieters R, Gallimore NE, Van Der Veer A, Meijerink JPP, Beverloo HB, et al. Outcome in children with Down's syndrome and acute lymphoblastic leukemia: Role of IKZF1 deletions and CRLF2 aberrations. Leukemia. 2012;26(10):2204-2211.

51. Zhou Y, Kanagal-Shamanna R, Zuo Z, Tang G, Medeiros LJ, Bueso-Ramos CE. Advances in B-lymphoblastic leukemia: cytogenetic and genomic lesions. Annals of Diagnostic Pathology. 2016;23:43-50.

52. Tasian SK, Doral MY, Borowitz MJ, Wood BL, Chen IM, Harvey RC, et al. Aberrant STAT5 and PI3K/mTOR pathway signaling occurs in human CRLF2-rearranged B-precursor acute lymphoblastic leukemia. Blood. 2012;120(4):833-842.

53. Mrózek K, Harper DP, Aplan PD. Cytogenetics and Molecular Genetics of Acute Lymphoblastic Leukemia. Hematology/Oncology Clinics of North America. 2009;23(5):991-v.

54. Zhang J, McCastlain K, Yoshihara H, Xu B, Chang Y, Churchman ML, et al. Deregulation of DUX4 and ERG in acute lymphoblastic leukemia. Nat Genet. 2016;48, 1481–1489

55. Gu Z, Churchman M, Roberts K, Li Y, Liu Y, Harvey RC, et al. Genomic analyses identify recurrent MEF2D fusions in acute lymphoblastic leukaemia. Nat Commun. 2016;7:13331.

56. Hirabayashi S, Ohki K, Nakabayashi K, Ichikawa H, Momozawa Y, Okamura K, et al. ZNF384-related fusion genes define a subgroup of childhood B-cell precursor acute lymphoblastic leukemia with a characteristic immunotype. Haematologica. 2017;102(1):118-129.

57. Liu YF, Wang BY, Zhang WN, Huang JY, Li BS, Zhang M, et al. Genomic Profiling of Adult and Pediatric B-cell Acute Lymphoblastic Leukemia. EBioMedicine. 2016;8:173-183.

58. Nebral K, Denk D, Attarbaschi A, König M, Mann G, Haas OA, et al. Incidence and diversity of PAX5 fusion genes in childhood acute lymphoblastic leukemia. Leukemia. 2009;23(1):134-143.

59. Moorman A V., Richards SM, Robinson HM, Strefford JC, Gibson BES, Kinsey SE, et al. Brief report: Prognosis of children with acute lymphoblastic leukemia (ALL) and intrachromosomal amplification of chromosome 21 (iAMP21). Blood. 2007; 109 (6): 2327–2330.

60. Rand V, Parker H, Russell LJ, Schwab C, Ensor H, Irving J, et al. Genomic characterization implicates iAMP21 as a likely primary genetic event in childhood B-cell precursor acute lymphoblastic leukemia. Blood. 2011;117(25):6848-6855.

61. Moorman A V., Robinson H, Schwab C, Richards SM, Hancock J, Mitchell CD, et al. Risk-directed treatment intensification significantly reduces the risk of relapse among children and adolescents with acute lymphoblastic leukemia and intrachromosomal amplification of chromosome 21: A comparison of the MRC ALL97/99 and UKALL2003 trials. J Clin Oncol. 2013;31(27):3389-3396.

62. Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature. 2007;446(7137):758-764.

63. Davis KL. Ikaros: Master of hematopoiesis, agent of leukemia. Therapeutic Advances in Hematology. 2011;2(6):359-368.

64. Belver L, Ferrando A. The genetics and mechanisms of T cell acute lymphoblastic leukaemia. Nature Reviews Cancer. 2016 ;16(8):494-507.

65. Stock W, Johnson JL, Stone RM, Kolitz JE, Powell BL, Wetzler M, et al. Dose intensification of daunorubicin and cytarabine during treatment of adult acute lymphoblastic leukemia: Results of Cancer and Leukemia Group B Study 19802. Cancer. 2013;120(14):2222.

66. Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC, et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. Cancer Cell. 2002;1(1):75-87.

67. Coustan-Smith E, Mullighan CG, Onciu M, Behm FG, Raimondi SC, Pei D, et al. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. Lancet Oncol. 2009;10(2):147-156.

68. Vlierberghe P Van, Ambesi-Impiombato A, Perez-Garcia A, Haydu JE, Rigo I, Hadler M, et al. ETV6 mutations in early immature human T cell leukemias. J Exp Med. 2011;208(13):2571-2579.

69. Niehues T, Kapaun P, Harms DO, Burdach S, Kramm C, Körholz D, et al. A classification based on T cell selection-related phenotypes identifies a subgroup of childhood T-ALL with favorable outcome in the COALL studies. Leukemia. 1999; 13, 614–617.

70. Girardi T, Vicente C, Cools J, De Keersmaecker K. The genetics and molecular biology of T-ALL. Blood. 2017;129(9):1113-1123.

71. Weng AP, Ferrando AA, Lee W, Morris IV JP, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science (80-). 2004;306(5694):269-271.

72. Dumortier A, Wilson A, MacDonald HR, Radtke F. Paradigms of notch signaling in mammals. International Journal of Hematology. 2005;82(4):277-284.

73. Ellisen,L W, Bird,J, West,D C, Soreng,A L, Reynolds,T C, Smith,S D, et al. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell. 1991;66(4):649-661.

74. Van Vlierberghe P, Ferrando A. The molecular basis of T cell acute lymphoblastic leukemia. Journal of Clinical Investigation. 2012;122(10):3398-3406.

75. Asnafi V, Buzyn A, Le Noir S, Baleydier F, Simon A, Beldjord K, et al. NOTCH1/FBXW7 mutation identifies a large subgroup with favorable outcome in adult T-cell acute lymphoblastic leukemia (T-ALL): A Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL) study. Blood. 2009;113(17):3918-3924.

76. Soulier J, Clappier E, Cayuela JM, Regnault A, García-Peydró M, Dombret H, et al. HOXA genes are included in genetic and biologic networks defining human acute T-cell leukemia (T-ALL). Blood. 2005;106(1):274-286.

77. AA F, DS N, RK D, Paietta E, RA L, PH W, et al. Prognostic importance of TLX1 (HOX11) oncogene expression in adults with T-cell acute lymphoblastic leukaemia. Lancet. 2004;363(9408):535-536.

78. Ballerini P, Blaise A, Busson-Le Coniat M, Su XY, Zucman-Rossi J, Adam M, et al. HOX11L2 expression defines a clinical subtype of pediatric T-ALL associated with poor prognosis. Blood. 2002;100(3):991-997.

79. Montaño A, Forero-Castro M, Marchena-Mendoza D, Benito R, Hernández-Rivas JM. New challenges in targeting signaling pathways in acute lymphoblastic leukemia by NGS approaches: An update. Cancers. 2018;10(4):110.

80. Neumann M, Vosberg S, Schlee C, Heesch S, Schwartz S, Gökbuget N, et al. Mutational spectrum of adult T-ALL. Oncotarget. 2015;6(5):2754-2766.

81. Kalender Atak Z, Gianfelici V, Hulselmans G, De Keersmaecker K, Devasia AG, Geerdens E, et al. Comprehensive Analysis of Transcriptome Variation Uncovers Known and Novel Driver Events in T-Cell Acute Lymphoblastic Leukemia. PLoS Genet. 2013;9(12):e1003997.

82. Vainchenker W, Constantinescu SN. JAK/STAT signaling in hematological malignancies. Oncogene. 2013;32(21):2601–13.

83. Shochat C, Tal N, Bandapalli OR, Palmi C, Ganmore I, te Kronnie G, et al. Gain-of-function mutations in interleukin-7 receptor-α (IL7R) in childhood acute lymphoblastic leukemias. J Exp Med. 2011;208(6):1333

84. Maude SL, Dolai S, Delgado-Martin C, Vincent T, Robbins A, Selvanathan A, et al. Efficacy of JAK/STAT pathway inhibition in murine xenograft models of early T-cell precursor (ETP) acute lymphoblastic leukemia. Blood. 2015;125(11):1759-1767.

85. Flex E, Petrangeli V, Stella L, Chiaretti S, Hornakova T, Knoops L, et al. Somatically acquired JAK1 mutations in adult acute lymphoblastic leukemia. J Exp Med. 2008;205(4):751–8.

86. Kontro M, Kuusanmäki H, Eldfors S, Burmeister T, Andersson EI, Bruserud, et al. Novel activating STAT5B mutations as putative drivers of T-cell acute lymphoblastic leukemia. Leukemia. 2014;28(8):1738-1742.

87. Schwaller J. Modeling ETV6-JAK2-induced leukemia: Insights from the zebrafish. Haematologica. 2012;97(12):1783-1785.

88. Todd MAM, Ivanochko D, Picketts DJ. Phf6 degrees of separation: The multifaceted roles of a chromatin adaptor protein. Genes. 2015.

89. Iacobucci I, Papayannidis C, Lonetti A, Ferrari A, Baccarani M, Martinelli G. Cytogenetic and molecular predictors of outcome in acute lymphocytic leukemia: Recent developments. Curr Hematol Malig Rep. 2012;6(2):325-352

90. Li B, Chng WJ. EZH2 abnormalities in lymphoid malignancies: Underlying mechanisms and therapeutic implications. Journal of Hematology and Oncology. 2019;12(1):118.

91. Ntziachristos P, Tsirigos A, Vlierberghe P Van, Nedjic J, Trimarchi T, Flaherty MS, et al. Genetic inactivation of the polycomb repressive complex 2 in T cell acute lymphoblastic leukemia. Nat Med. 2012;18(2):298-301.

92. Grossmann V, Haferlach C, Weissmann S, Roller A, Schindela S, Poetzinger F, et al. The molecular profile of adult T-cell acute lymphoblastic leukemia: Mutations in RUNX1 and DNMT3A are associated with poor prognosis in T-ALL. Genes Chromosom Cancer. 2013;54(10):653

93. Alvarnas JC, Brown PA, Aoun P, Ballen KK, Barta SK, Borate U, et al. Acute Lymphoblastic Leukemia, Version 2.2015. J Natl Compr Cancer Netw. 2015;13(10):1240-1279.

94. Rowe JM. Prognostic factors in adult acute lymphoblastic leukaemia. British Journal of Haematology. 2010;150(4):389-405.

95. Lee JW, Cho B. Prognostic factors and treatment of pediatric acute lymphoblastic leukemia. Korean Journal of Pediatrics. 2017;60(5):129-137..

96. Inaba H, Greaves M, Mullighan CG. Acute lymphoblastic leukaemia. Lancet. 2013;381(9881):1943–55.

97. Starza I Della, Chiaretti S, De Propris MS, Elia L, Cavalli M, De Novi LA, et al. Minimal residual disease in acute lymphoblastic leukemia: Technical and clinical advances. Front Oncol. 2019;9(August):1–17.

98. Campana D. Minimal residual disease in acute lymphoblastic leukemia. Hematology Am Soc Hematol Educ Program. 2010;2010:7-12.

99. Bruggemann M, Schrauder A, Raff T, Pfeifer H, Dworzak M, Ottmann OG, et al. Standardized MRD quantification in European all trials: Proceedings of the second international symposium on MRD assessment in Kiel, Germany, 18-20 September 2008. Leukemia. 2010;24(3):521-535.

100. Gökbuget N, Dombret H, Giebel S, Bruggemann M, Doubek M, Foà R, et al. Minimal residual disease level predicts outcome in adults with Ph-negative B-precursor acute lymphoblastic leukemia. Hematol (United Kingdom). 2019;24(1):337-348.

101. Brüggemann M, Gökbuget N, Kneba M. Acute lymphoblastic leukemia: Monitoring minimal residual disease as a therapeutic principle. Semin Oncol. 2012;39(1):47-57.

102. Stock W. Adolescents and young adults with acute lymphoblastic leukemia. Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program. 2010; 2010 (1): 21–29.

103. Huguet F, Leguay T, Raffoux E, Thomas X, Beldjord K, Delabesse E, et al. Pediatric-inspired therapy in adults with philadelphia chromosome-negative acute lymphoblastic leukemia: The GRAALL-2003 study. J Clin Oncol. 2009;27(15):2574.

104. Stanulla M, Schrappe M. Treatment of Childhood Acute Lymphoblastic Leukemia. Semin Hematol. 2009;46(1):52-63.

105. Bassan R, Hoelzer D. Modern therapy of acute lymphoblastic leukemia. Journal of Clinical Oncology. 2011;29(5):532-543.

106. Narayanan S, Shami PJ. Treatment of acute lymphoblastic leukemia in adults. Critical Reviews in Oncology/Hematology. 2012 ;81(1):94-102.

107. Cooper SL, Brown PA. Treatment of pediatric acute lymphoblastic leukemia. Pediatric Clinics of North America. 2015;62(1):61-73

108. Larson RA, Dodge RK, Burns CP, Lee EJ, Stone RM, Schulman P, et al. A five-drug remission induction regimen with intensive consolidation for adults with acute lymphoblastic leukemia: Cancer and leukemia group B study 8811. Blood. 1995;85(8):2025-2037.

109. Rowe JM, Buck G, Burnett AK, Chopra R, Wiernik PH, Richards SM, et al. Induction therapy for adults with acute lymphoblastic leukemia: Results of more than 1500 patients from the international ALL trial: MRC UKALL XII/ECOG E2993. Blood. 2005;106(12):3760-3767.

110. Kantarjian HM, O'Brien S, Smith TL, Cortes J, Giles FJ, Beran M, et al. Results of treatment with hyper-CVAD, a dose-intensive regimen, in adult acute lymphocytic leukemia. J Clin Oncol. 2000;18(3):547-561.

111. Thomas DA, O'Brien SM, Faderl S, Ravandi Kashani F, Wierda WG, Andreeff M, et al. Long-term outcome after hyper-CVAD and imatinib (IM) for de novo or minimally

treated Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph-ALL). J Clin Oncol. 2010;28:15_suppl, 6506-6506

112. Ravandi F, O'Brien SM, Cortes JE, Thomas DM, Garris R, Faderl S, et al. Long-term follow-up of a phase 2 study of chemotherapy plus dasatinib for the initial treatment of patients with Philadelphia chromosome-positive acute lymphoblastic leukemia. Cancer. 2015;121(23):4158-4164.

113. Cortes JE, Kim DW, Pinilla-Ibarz J, Le Coutre P, Paquette R, Chuah C, et al. A phase 2 trial of ponatinib in Philadelphia chromosome-positive leukemias. N Engl J Med. 2013;369(19):1783-1796.

114. Ravandi F, O'Brien S, Thomas D, Faderl S, Jones D, Garris R, et al. First report of phase 2 study of dasatinib with hyper-CVAD for the frontline treatment of patients with Philadelphia chromosome-positive (Ph +) acute lymphoblastic leukemia. Blood. 2010;116(12):2070-2077.

115. Faderl S, Thomas DA, O'Brien S, Ravandi F, Garcia-Manero G, Borthakur G, et al. Augmented hyper-CVAD based on dose-intensified vincristine, dexamethasone, and asparaginase in adult acute lymphoblastic leukemia salvage therapy. Clin Lymphoma, Myeloma Leuk. 2011;11(1):54-59.

116. Shah NN, Stevenson MS, Yuan CM, Richards K, Delbrook C, Kreitman RJ, et al. Characterization of CD22 expression in acute lymphoblastic leukemia. Pediatr Blood Cancer. 2015;62(6):964-969.

117. Piccaluga PP, Arpinati M, Candoni A, Laterza C, Paolini S, Gazzola A, et al. Surface antigens analysis reveals significant expression of candidate targets for immunotherapy in adult acute lymphoid leukemia. Leukemia and Lymphoma. 2011;52(2):325-327.

118. Hinman LM, Hamann PR, Wallace R, Menendez AT, Durr FE, Upeslacis J. Preparation and Characterization of Monoclonal Antibody Conjugates of the Calicheamicins: A Novel and Potent Family of Antitumor Antibiotics. Cancer Res. 1993;53(14):3336-3342.

119. Messinger YH, Gaynon PS, Sposto R, Van Der Giessen J, Eckroth E, Malvar J, et al. Bortezomib with chemotherapy is highly active in advanced B-precursor acute

lymphoblastic leukemia: Therapeutic Advances in Childhood Leukemia & Lymphoma (TACL) Study. Blood. 2012;120(2):285-290.

120. Lyko F, Brown R. DNA methyltransferase inhibitors and the development of epigenetic cancer therapies. Journal of the National Cancer Institute. 2005;1498-1506

121. Rahmani M, Talebi M, Hagh MF, Feizi AAH, Solali S. Aberrant DNA methylation of key genes and Acute Lymphoblastic Leukemia. Biomedicine and Pharmacotherapy. 2018;1493-1500

122. Bongiovanni D, Saccomani V, Piovan E. Aberrant Signaling Pathways in T-Cell Acute Lymphoblastic Leukemia. Int J Mol Sci. 2017;18(9):1904.

123. Forero-Castro M, Robledo C, Benito R, Bodega-Mayor I, Rapado I, Hernández-Sánchez M, et al. Mutations in TP53 and JAK2 are independent prognostic biomarkers in B-cell precursor acute lymphoblastic leukaemia. Br J Cancer. 2017;117(2):256-265.

124. N. J, E.J. J, P.Z. M, F. R, K. T, T. K, et al. Ruxolitinib or dasatinib in combination with chemotherapy for patients with relapsed/refractory philadelphia (PH)-like acute lymphoblastic leukemia: A phase I-II trial. Blood. 2017;130 (Supplement 1): 1322.

125. Rawlings JS. The JAK/STAT signaling pathway. J Cell Sci. 2004;117(8):1281–3.

126. Schwartzman O, Savino AM, Gombert M, Palmi C, Cario G, Schrappe M, et al. Suppressors and activators of JAK-STAT signaling at diagnosis and relapse of acute lymphoblastic leukemia in Down syndrome. Proc Natl Acad Sci U S A. 2017;114(20):E4030-E4039.

127. Yamaoka K, Saharinen P, Pesu M, Et V, Iii H, Silvennoinen O, et al. Protein family review The Janus kinases (Jaks). Genome Biol. 2004;5(253).

128. Musso T, Johnston JA, Linnekin D, Varesio L, Rowe TK, O'Shea JJ, et al. Regulation of JAK3 expression in human monocytes: Phosphorylation in response to interleukins 2, 4, and 7. J Exp Med. 1995;181(4):1425-1431.

129. O'Shea JJ, Holland SM, Staudt LM. JAKs and STATs in immunity, immunodeficiency, and cancer. New England Journal of Medicine. 2013 ;368(2):161-170.

130. Hosokawa M, Satoh T. Structure, function, and regulation of carboxylesterases. Toxicol Organophosphate Carbamate Compd. 2006;219–31.

131. Langenfeld F, Guarracino Y, Arock M, Trouvé A, Tchertanov L. How Intrinsic Molecular Dynamics Control Intramolecular Communication in Signal Transducers and Activators of Transcription Factor STAT5. PLoS One. 2015;10(12).

132. Bousoik E, Montazeri Aliabadi H. "Do We Know Jack" About JAK? A Closer Look at JAK/STAT Signaling Pathway. Front Oncol. 2018;8:287.

133. Furqan M, Mukhi N, Lee B, Liu D. Dysregulation of JAK-STAT pathway in hematological malignancies and JAK inhibitors for clinical application. Biomark Res. 2013;1(1):5.

134. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene. 2002;285(1–2):1–24.

135. Lohi O, Lehto VP. STAM/EAST/Hbp adapter proteins - Integrators of signalling pathways. FEBS Letters. 2001;508(3):287-290.

136. Greenhalgh CJ, Hilton DJ. Negative regulation of cytokine signaling. J Leukoc Biol. 2001;70(3):348-356.

137. Kiu H, Nicholson SE. Biology and significance of the JAK/STAT signalling pathways. Growth Factors. 2012;30(2):88-106.

138. Niu GJ, Xu JD, Yuan WJ, Sun JJ, Yang MC, He ZH, et al. Protein inhibitor of activated STAT (PIAS) negatively regulates the JAK/STAT pathway by inhibiting STAT phosphorylation and translocation. Front Immunol. 2018;9:2392.

139. Igaz P, Toth S, Falus A. Biological and clinical significance of the JAK-STAT pathway; lessons from knockout mice. Inflamm Res. 2001;50(9):435–41-

140. Alexander DR. The CD45 tyrosine phosphatase: A positive and negative regulator of immune cell function. Semin Immunol. 2000;12(4):349-359.

141. Irie-Sasaki J, Sasaki T, Matsumoto W, Opavsky A, Cheng M, Welstead G, et al. CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. Nature. 2001;409, 349–354.

142. Babon JJ, Lucet IS, Murphy JM, Nicola NA, Varghese LN. The molecular regulation of Janus kinase (JAK) activation. Biochemical Journal. 2014;462(1):1-13.

143. Levine RL, Pardanani A, Tefferi A, Gilliland DG. Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders. Nature Reviews Cancer. 2007;7(9):673-683.

144. Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene. 2007;26(22):3291-3310.

145. Porta C, Paglino C, Mosca A. Targeting PI3K/Akt/mTOR signaling in cancer. Frontiers in Oncology. 2014;4:64.

146. Churchman ML, Evans K, Richmond J, Robbins A, Jones L, Shapiro IM, et al. Synergism of FAK and tyrosine kinase inhibition in Ph+ B-ALL. JCI Insight. 2016;1(4):e86082.

147. Knight T, Irving JAE. Ras/Raf/MEK/ERK pathway activation in childhood acute lymphoblastic leukemia and its therapeutic targeting. Frontiers in Oncology. 2014;4:160.

148. Harrison CJ. Targeting signaling pathways in acute lymphoblastic leukemia: new insights. Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program. 2013; 2013 (1): 118–125.

149. Mullighan CG, Collins-Underwood JR, Phillips LAA, Loudin MG, Liu W, Zhang J, et al. Rearrangement of CRLF2 in B-progenitor-and Down syndrome-associated acute lymphoblastic leukemia. Nat Genet. 2009;41(11):1243-1246.

150. Bains T, Heinrich MC, Loriaux MM, Beadling C, Nelson D, Warrick A, et al. Newly described activating JAK3 mutations in T-cell acute lymphoblastic leukemia. Leukemia. 2012;26(9):2144-2146.

151. Onnebo SMN, Rasighaemi P, Kumar J, Liongue C, Ward AC. Alternative TEL-JAK2 fusions associated with T-cell acute lymphoblastic leukemia and atypical chronic myelogenous leukemia dissected in zebrafish. Haematologica. 2012;97(12):1895-1903.

152. Bandapalli OR, Schuessele S, Kunz JB, Rausch T, Stütz AM, Tal N, et al. The activating STAT5B N642H mutation is a common abnormality in pediatric T-cell

acutelymphoblastic leukemia and confers a higher risk of relapse. Haematologica. 2014;99(10):e188-e192.

153. Gouilleux-Gruart V, Gouilleux F, Desaint C, Claisse JF, Capiod JC, Delobel J, et al. STAT-related transcription factors are constitutively activated in peripheral blood cells from acute leukemia patients. Blood. 1996;87(5):1692-1697.

154. Brachet-Botineau M, Polomski M, Neubauer HA, Juen L, Hédou D, Viaud-Massuard MC, et al. Pharmacological inhibition of oncogenic STAT3 and STAT5 signaling in hematopoietic cancers. Cancers. 2020;12(1):240.

155. Michalak M, Groenendyk J, Szabo E, Gold LII, Opas M. Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. Biochem J. 2009;417(3):651–66.

156. Michalak M, Corbett EF, Mesaeli N, Nakamura K, Opas M. Calreticulin: One protein, one gene, many functions. Biochemical Journal. 1999;344 Pt 2(Pt 2):281-292.

157. Lee H, Lu Y, Weng W, Lee H. Functional Roles of Calreticulin in Cancer Biology Review Article Functional Roles of Calreticulin in Cancer Biology. Biomed Res Int. 2015;2015(April).

158. Mendlovic F, Conconi M. Calreticulin: a Multifaceted Protein. Nat Educ. 2011;

159. Machado-Neto JA, Campos P de M, Trainaen F. CALR (calreticulin). Atlas Genet Cytogenet Oncol Haematol. 2018;

160. Nakamura K, Zuppini A, Arnaudeau S, Lynch J, Ahsan I, Krause R, et al. Functional specialization of calreticulin domains. J Cell Biol. 2001;154(5):961-972.

161. Greber UF, Gerace L. Depletion of calcium from the lumen of endoplasmic reticulum reversibly inhibits passive diffusion and signal-mediated transport into the nucleus. J Cell Biol. 1995;128(1-2):5-14.

162. Ashby MC, Tepikin A V. ER calcium and the functions of intracellular organelles. Semin Cell Dev Biol. 2001;12(1):11-17.

163. Chiang WF, Hwang TZ, Hour TC, Wang LH, Chiu CC, Chen HR, et al. Calreticulin, an endoplasmic reticulum-resident protein, is highly expressed and essential for cell

proliferation and migration in oral squamous cell carcinoma. Oral Oncol. 2013;49(6):534-541.

164. Du XL, Yang H, Liu SG, Luo ML, Hao JJ, Zhang Y, et al. Calreticulin promotes cell motility and enhances resistance to anoikis through STAT3-CTTN-Akt pathway in esophageal squamous cell carcinoma. Oncogene. 2009;28(42):3714-3722.

165. Shi F, Shang L, Pan BQ, Wang XM, Jiang YY, Hao JJ, et al. Calreticulin promotes migration and invasion of esophageal cancer cells by upregulating neuropilin-1 expression via STAT5A. Clin Cancer Res. 2014;20(23):6153-6162.

166. Wang XM, Shang L, Zhang Y, Hao JJ, Shi F, Luo W, et al. PTP1B contributes to calreticulin-induced metastatic phenotypes in esophageal squamous cell carcinoma. Mol Cancer Res. 2013;11(9):986-994.

167. Feng R, Ye J, Zhou C, Qi L, Fu Z, Yan B, et al. Calreticulin down-regulation inhibits the cell growth, invasion and cell cycle progression of human hepatocellular carcinoma cells. Diagn Pathol. 2015;10:149.

168. Raghavan M, Wijeyesakere SJ, Peters LR, Del Cid N. Calreticulin in the immune system: Ins and outs. Trends Immunol. 2013;34(1):13–21.

169. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini J-L, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med. 2007;13(1):54–61.

170. Chao MP, Jaiswal S, Weissman-Tsukamoto R, Alizadeh AA, Gentles AJ, Volkmer J, et al. Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. Sci Transl Med. 2010;2(63):63ra94.

171. Varricchio L, Falchi M, Dall'Ora M, De Benedittis C, Ruggeri A, Uversky VN, et al. Calreticulin: Challenges posed by the intrinsically disordered nature of calreticulin to the study of its function. Front Cell Dev Biol. 2017;5:96.

172. Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD, et al. Somatic Mutations of Calreticulin in Myeloproliferative Neoplasms. N Engl J Med. 2013;369(25):2379–90.

173. Nangalia J, Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC, et al. Somatic CALR Mutations in Myeloproliferative Neoplasms with Nonmutated JAK2. N Engl J Med. 2013;369(25):2391–405.

174. Kjaer L, Holmström MO, Cordua S, Andersen MH, Svane IM, Thomassen M, et al. Sorted peripheral blood cells identify CALR mutations in B- and T-lymphocytes. Leuk Lymphoma. 2017;0(0):1–5.

175. Wang J, Hao J, He N, Ji C, Ma D. The Mutation Profile of Calreticulin in Patients with Myeloproliferative Neoplasms and Acute Leukemia. Turkish J Hematol. 2016 ;33(3):180-186.

176. Rumi E, Pietra D, Ferretti V, Klampfl T, Harutyunyan AS, Milosevic JD, et al. JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. Blood. 2014;123(10):1544–51.

177. Chachoua I, Pecquet C, El-khoury M, Nivarthi H, Albu R, Marty C, et al. Thrombopoietin receptor activation by myeloproliferative neoplasm associated calreticulin mutants. 2018;127(10):1325–36.

178. Araki M, Yang Y, Masubuchi N, Hironaka Y, Takei H, Morishita S, et al. Activation of the thrombopoietin receptor by mutant calreticulin in CALR-mutant myeloproliferative neoplasms. Blood. 2016;127(10):1307-1316.

179. Balligand T, Achouri Y, Pecquet C, Chachoua I, Nivarthi H, Marty C, et al. Pathologic activation of thrombopoietin receptor and JAK2-STAT5 pathway by frameshift mutants of mouse calreticulin. Leukemia. 2016;30(8):1775-1778.

180. Burns EA, Anand K, Chung B, Shah S, Randhawa JK, Pingali SR. The development of T-cell malignancies in patients with pre-existing myeloproliferative neoplasms: a report of three cases. Ecancermedicalscience. 2020;14:1011.

181. Langabeer SE, Haslam K, Brien DO, Kelly J, Andrews C, Ryan C, et al. Case Report Acute Lymphoblastic Leukemia Arising in CALR Mutated Essential Thrombocythemia. 2016;2016.6(1):155-161.

182. Ji C, Lin S, Yao D, Li M, Chen W, Zheng S, et al. Identification of promising prognostic genes for relapsed acute lymphoblastic leukemia. Blood Cells, Mol Dis. 2019;77:113-119.

183. Li C, Kuang L, Zhu B, Chen J, Wang X, Huang X. Identification of prognostic risk factors of acute lymphoblastic leukemia based on mRNA expression profiling. Neoplasma. 2017;64(4):494-501.

184. Vainchenker W, Leroy E, Gilles L, Marty C, Plo I, Constantinescu SN. JAK inhibitors for the treatment of myeloproliferative neoplasms and other disorders. F1000Research. 2018;7:82.

185. Tasian SK, Assad A, Hunter DS, Du Y, Loh ML. A Phase 2 Study of Ruxolitinib with Chemotherapy in Children with Philadelphia Chromosome-like Acute Lymphoblastic Leukemia (INCB18424-269/AALL1521): Dose-Finding Results from the Part 1 Safety Phase. Blood. 2018;132 (Supplement 1): 555.

186. Ding YY, Stern JW, Jubelirer TF, Wertheim GB, Lin F, Chang F, et al. Clinical efficacy of ruxolitinib and chemotherapy in a child with philadelphia chromosome-like acute lymphoblastic leukemia with GOLGA5-JAK2 fusion and induction failure. Haematologica. 2018;103(9):e427-e431.

187. Bhalla S, Isola L, Steinberg A. First Description of Ruxolitinib in JAK2+ ALL: Institutional Experience with Noted Response. Biol Blood Marrow Transplant. 2017;23:S244-S245

188. Fielding AK, Richards SM, Chopra R, Lazarus HM, Litzow MR, Buck G, et al. Outcome of 609 adults after relapse of acute lymphoblastic leukemia (ALL); an MRC UKALL12/ECOG 2993 study. Blood. 2007;109(3):944-950.

189. Ebadi M, Wasko J, Weisdorf DJ, Gordon PM, Rashidi A. Ruxolitinib combined with chemotherapy can eradicate chemorefractory central nervous system acute lymphoblastic leukaemia. British Journal of Haematology. 2019 ;187(1):e24-e27.

190. National Center for Biotechnology Information. In 2019.

191. Pringsheim T, Marras C. Pimozide for tics in Tourette's syndrome. Cochrane Database of Systematic Reviews. 2009;2009(2):CD006996.

192. Mothi M, Sampson S. Pimozide for schizophrenia or related psychoses. Cochrane Database of Systematic Reviews. 2013;(3):CD001949.

193. Nelson EA, Walker SR, Weisberg E, Bar-Natan M, Barrett R, Gashin LB, et al. The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors. Blood. 2011;117(12):3421-3429.

194. Rondanin R, Simoni D, Romagnoli R, Baruchello R, Marchetti P, Costantini C, et al. Inhibition of activated STAT5 in Bcr/Abl expressing leukemia cells with new pimozide derivatives. Bioorganic Med Chem Lett. 2014;24(18):4568-4574.

195. Bar-Natan M, Nelson EA, Walker SR, Kuang Y, Distel RJ, Frank DA. Dual inhibition of Jak2 and STAT5 enhances killing of myeloproliferative neoplasia cells. Leukemia. 2012;26(6):1407–10.

196. Findley HW, Cooper MD, Kim TH, Alvarado C, Ragab AH. Two new acute lymphoblastic leukemia cell lines with early B-cell phenotypes. Blood. 1982;60(6):1305-1309.

197. Perram, C.M, Nicolau, C PJW. Phenotypic characterization of a unique non-T, non-B acute lymphoblastic leukaemia cell line. Nature. 1977;270(December 15):572.

198. Matsuo Y, Drexler HG. Establishment and characterization of human B cell precursor-leukemia cell lines. Leuk Res. 1998;22(7):567–79.

199. Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA, McCarthy RE. Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. Cancer. 1965;18(4):522–9.

200. Nagel S, Kaufmann M, Drexler HG, MacLeod RAF. The cardiac homeobox gene NKX2-5 is deregulated by juxtaposition with BCL11B in pediatric T-ALL cell lines via a novel t(5;14)(q35.1;q32.2). Cancer Res. 2003;63(17):5329-5334.

201. Schneider U, Schwenk H -U, Bornkamm G. Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic

leukemia and leukemic transformed non-Hodgkin lymphoma. Int J Cancer. 1977;19(5):621–6.

202. Communication B, Lymphoid RH, Lines C. Rosette-Forming Human Lymphoid Cell Lines. 1971;(August 1970):891–5.

203. Strober W. Trypan Blue Exclusion Test of Cell Viability. In: Current Protocols in Immunology. 2001. 21: A.3B.1-A.3B.2.

204. Chomczynski P. Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. 1987;159:156–9.

205. Gomes A, Korf MSB. Genetic Testing Techniques. Pediatric Cancer Genetics. Elsevier Inc.; 47–64 p.

206. Rampersad SN. Multiple applications of alamar blue as an indicator of metabolic function and cellular health in cell viability bioassays. Sensors (Switzerland). 2012;12(9):12347–60.

207. Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: basic principles and applications. Crit Rev Biotechnol. 2017;37(2):163–76.

208. Kerr JFR, Winterford CM, Harmon B V. Apoptosis. Its significance in cancer and cancer Therapy. Cancer. 1994;73(8):2013–26.

209. Aubry JP, Blaecke A, Lecoanet-Henchoz S, Jeannin P, Herbault N, Caron G, et al. Annexin V used for measuring apoptosis in the early events of cellular cytotoxicity. Cytometry. 1999;37(3):197–204.

210. George TC, Basiji DA, Hall BE, Lynch DH, Ortyn WE, Perry DJ, et al. Distinguishing modes of cell death using the ImageStream® multispectcal imaging flow cytometer. Cytom Part A. 2004;59(2):237–45.

211. Zembruski NCL, Stache V, Haefeli WE, Weiss J. 7-Aminoactinomycin D for apoptosis staining in flow cytometry. Anal Biochem. 2012;429(1):79–81.

212. Jayat C, Ratinaud MH. Cell cycle analysis by flow cytometry: Principles and applications. Biol Cell. 1993;78(1–2):15–25.

213. Brown M, Wittwer C. Flow Cytometry: Principles and Clinical Applications in Hematology. Clin Chem. 2000;46(8(B)):1221–9.

214. Pozarowski P, Darzynkiewicz Z. Analysis of cell cycle by flow cytometry. Methods Mol Biol. 2004;281:301–11.

215. Aldoss IT, Marcucci G, Pullarkat V. Treatment of acute lymphoblastic leukemia in adults: Applying lessons learned in children. ONCOLOGY (United States). 2016.;30(12):1080-1091.

216. Mullighan CG, Zhang J, Harvey RC, Collins-Underwood JR, Schulman BA, Phillips LA, et al. JAK mutations in high-risk childhood acute lymphoblastic leukemia. Proc Natl Acad Sci. 2009;106(23):9414–8.

217. Walker KL, Kabakov SA, Zhu F, Bouchlaka MN, Olson SL, Cho MM, et al. Efficacy of JAK1/2 and BCL2 inhibition on human T cell acute lymphoblastic leukemia in vitro and in vivo. bioRxiv. 2019 Jan 1;734913.

218. Zhang Q, Shi C, Han L, Jain N, Roberts KG, Ma H, et al. Inhibition of mTORC1/C2 signaling improves anti-leukemia efficacy of JAK/STAT blockade in CRLF2 rearranged and/or JAK driven philadelphia chromosome-like acute B-cell lymphoblastic leukemia. Oncotarget. 2018; 9: 8027-8041.

219. Inthal A, Krapf G, Beck D, Joas R, Kauer MO, Orel L, et al. Role of the erythropoietin receptor in ETV6/RUNX1-positive acute lymphoblastic leukemia. Clin Cancer Res. 2008;14(22):7196-7204.

220. Torrano V, Procter J, Cardus P, Greaves M, Ford AM. ETV6-RUNX1 promotes survival of early B lineage progenitor cells via a dysregulated erythropoietin receptor. Blood. 2011;118(18):4910-4918.

221. Sun C, Chang L, Zhu X. Pathogenesis of ETV6/RUNX1-positive childhood acute lymphoblastic leukemia and mechanisms underlying its relapse. Oncotarget. 2017;8(21):35445-35459.

222. Eastman A. Improving anticancer drug development begins with cell culture: misinformation perpetrated by the misuse of cytotoxicity assays. Oncotarget. 2017;8(5):8854-8866.

223. Zhou T, Georgeon S, Moser R, Moore DJ, Caflisch A, Hantschel O. Specificity and mechanism-of-action of the JAK2 tyrosine kinase inhibitors ruxolitinib and SAR302503 (TG101348). Leukemia. 2014;28, 404–407

224. Duque-Afonso J, Feng J, Scherer F, Lin CH, Wong SHK, Wang Z, et al. Comparative genomics reveals multistep pathogenesis of E2A-PBX1 acute lymphoblastic leukemia. J Clin Invest. 2015;125(9):3667-3680.

225. Nagy ZS, Rui H, Stepkowski SM, Karras J, Kirken RA. A Preferential Role for STAT5, not Constitutively Active STAT3, in Promoting Survival of a Human Lymphoid Tumor. J Immunol. 2006;177(8):5032-5040.

226. Machado-Neto, J.A., Coelho-Silva, J.L., Santos, F.P.d. et al. Autophagy inhibition potentiates ruxolitinib-induced apoptosis in JAK2V617F cells. Invest New Drugs. 2020; 38, 733–745 (2020).

227. Kinzler, M.N., Zielke, S., Kardo, S. et al. STF-62247 and pimozide induce autophagy and autophagic cell death in mouse embryonic fibroblasts. Sci Rep. 2020; 10, 687.

228. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol. 2010;22(2):124-131.

229. Kong Y, Wu YL, Song Y, Shi MM, Cao XN, Zhao HY, et al. Ruxolitinib/nilotinib cotreatment inhibits leukemia-propagating cells in Philadelphia chromosome-positive ALL. J Transl Med. 2017;15(1):184.

230. Nelson EA, Walker SR, Xiang M, Weisberg E, Bar-Natan M, Barrett R, et al. The STAT5 Inhibitor Pimozide Displays Efficacy in Models of Acute Myelogenous Leukemia Driven by FLT3 Mutations. Genes and Cancer. 2012; ;3(7-8):503-511.

231. National Center for Biotechnology Information. PubChem Database. Ruxolitinib, CID=25126798.

232. National Center for Biotechnology Information. PubChem Database. Pimozide, CID=16362.