UNIVERSITÀ
DEGLI STUDI
DI PADOVA

DIPARTIMENTO DI PEDIATRIA "*SALUS PUERI*"

SCUOLA DI DOTTORATO DI RICERCA

in Medicina dello Sviluppo e Scienze della Programmazione

Indirizzo Ematooncologia, Immunologia e Genetica

XXIV CICLO

Lack of Protein Kinase C alpha is associated with poor prognosis in pediatric T-ALL: a phosphoproteomic discovery

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2009-2011

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SUMMARY

Acute Lymphoblastic Leukemia (ALL), the most common pediatric cancer, is an aggressive malignancy of lymphopoietic cells characterized by a clonal proliferation of blast cells originated from lymphoid precursors arrested at early stages of differentiation. Nowadays the introduction of risk-directed treatment and intensified cure protocols has improved the outcome of ALL pediatric patients. Although current therapies achieve five-year event-free survival rate of about 80% in children, the rest of patients experience treatment resistance and risk of early relapse. In order to develop new additional therapeutic supports and to assemble specific and personalized therapies, improving drug response and reducing drug toxicity, it is extremely important to identify novel therapeutic targets and new prognostic biomarkers on leukemic cells.

To pursue this aim, Reverse Phase Protein Arrays (RPPA) approach represents a powerful tool to profile protein pathways in order to identify novel diagnostic and prognostic biomarkers. RPPA can measure the activation levels/phosphorylation of large numbers of signalling proteins from small clinical samples in a very reproducible, precise, sensitive and high-throughput manner. The identification of critical nodes or interactions within the protein network is a potential starting point for drug development and/or design of individual therapy regimens.

In RPPA, protein lysates are immobilized on a nitrocellulose-coated glass slide. Each group of spots represents an individual test sample, such that an array is comprised of multiple, different samples. Each RPPA slide is probed with a single detection molecule (one slide = one antibody) and a single analyte is concurrently measured across multiple samples. This format allows multiple samples to be analyzed under the same experimental conditions for any given analyte. This

approach facilitates comparisons between samples, since experimental variability is eliminated. During my PhD research I applied the Reverse Phase Protein Arrays (RPPA) technique to the study of patients affected by Acute Leukemia.

The principal aim of this research was the discovery of new candidate biomarkers and therapeutic targets involved in the leukemic process through the study of the phosphoproteomic profiles of ALL pediatric patients (T-ALL and B-ALL) by means of the RPPA technique.

In the study of pediatric T-ALL at diagnosis reported in the "Main Topic", we identified PKCα as a new prognostic marker of relapse. This promising protein kinase has been identified through a retrospective RPPA screening of fifty-three different proteins/phophoproteins in 98 T-ALL specimens. PKCα resulted to be downregulated in the group of patients with a higher incidence of relapse. We showed that low PKCα activation is a consequence of a low *PKCα* gene expression. Moreover, we induced PKCα inhibition in T-ALL cell lines using a PKCα commercial inhibitor and an increase in proliferation rate was detected. RPPA thus proved to be a useful approach to discover new molecules aberrantly expressed in leukemia pathways. The identification of players involved in malignant mechanisms allows focusing on novel prognostic factors: PKCα resulted to be a promising marker of risk of relapse in pediatric T-ALL. A study of PKCα expression in a larger T-ALL patients cohort should confirm this relevant data and to propose PKCα as a new prognostic marker for T-ALL patients stratification.

In the pediatric B-cell precursor (BCP)-ALL study we used RPPA to map in 118 pediatric BCP-ALL patients for the expression/activation of 92 different proteins/phophoproteins part of key signalling "hubs". Correlation of signalling activation with clinical response and known genetic information enabled us to identify new protein pathway biomarkers that, when validated in larger clinical sets, could be used for patients stratification and targeted therapy trials. In detail, we observed an increased activation/expression of several pathways involved in cell proliferation in patients with a clinical poor phenotype. First, MLL-rearranged

leukemia revealed BCL-2 hyperphosphorylation caused by AMPK activation, indicating that AMPK could be responsible of apoptosis inhibition in MLL-rearranged patients and thus could be considered as a new potential therapeutic target. Second, in patients with poor clinical response to Prednisone we observed the up-modulation of LCK activity with respect to patients with good response. This tyrosine-kinase can be down-modulated with clinically used inhibitors that could be considered for further studies as a new additional therapy for Prednisone-resistant patients. Further we also found an association between high levels of CYCLIN E and relapse incidence. Moreover, CYCLIN E is more expressed in patients with early relapsed, who usually show an unfavourable prognosis. We decided to explore in deep the functional significance of AMPK activation in MLL-rearranged patients. We showed that the AMPK pathway contributes directly to the survival of MLL-rearranged BCP-ALL cells and AMPK can be considered a new druggable target in MLL-rearranged leukemias.

RPPA reliability in detection of novel targets and markers has been demonstrated also in collaborative studies with national and international research groups, regarding also adult diseases such as Acute Myeloid Leukemia.

In my PhD research I showed that the application of Reverse Phase Protein Arrays is a reliable, sensitive and high-throughput method to identify new aberrant molecular players involved in haematological malignancies. These findings will be useful in order to discover novel prognostic markers and therapeutic targets to propose for the development of more effective patient-tailored treatments.

RIASSUNTO

La Leucemia Linfoblastica Acuta (LAL), la forma di tumore pediatrico più diffusa, è una patologia delle cellule linfopoietiche caratterizzata da una proliferazione clonale dei blasti originati dai precursori linfoidi bloccati a stati differenziativi precoci. Ad oggi l'introduzione di trattamenti differenziati sulla base del rischio di ricaduta e di protocolli di cura più intensi ha portato ad un miglioramento della prognosi dei pazienti affetti da LAL. Anche se le terapie attualmente usate permettono di ottenere una sopravvivenza dell'80% a 5 anni dalla diagnosi, un paziente ogni cinque presenta resistenza alla terapia e rischio di ricaduta precoce. E' dunque fondamentale identificare nuovi target terapeutici e nuovi biomarcatori delle cellule leucemiche al fine di sviluppare nuovi trattamenti di supporto e di individuare cure più specifiche, migliorando la risposta alle terapie e riducendo la loro tossicità. Per perseguire tale obiettivo, l'approccio sperimentale dei Reverse Phase Protein Arrays (RPPA) rappresenta un potente mezzo per studiare il profilo proteico delle vie di trasduzione del segnale con lo scopo di identificare nuovi marker prognostici o target terapeutici. Con la metodica degli RPPA è possibile misurare il livello di attivazione/espressione di un ampio numero di fosfoproteine/proteine partendo da limitate quantità di campione in maniera riproducibile, precisa, sensibile e *high througput*. L'identificazione di alterazioni nei punti chiave dei network delle cellule tumorali rappresenta un potenziale punto di partenza per lo sviluppo di nuovi farmaci o l'ideazione di nuovi regimi terapeutici.

Nel metodo degli RPPA, il proteoma di un set di campioni viene immobilizzato su un vetrino ricoperto di nitrocellulosa. Ciascun gruppo di spot rappresenta un signolo campione in analisi e nello stesso vetrino è possibile studiare simultaneamente fino a cento campioni. Ciascun vetrino viene poi marcato con un

anticorpo: in tal modo ogni singolo analita viene sottoposto alle stesse condizioni di marcatura e tale riduzione nella variabilità sperimentale facilita il confronto tra diversi campioni.

Durante il mio dottorato di ricerca ho applicato la tecnica dei Reverse Phase Protein Arrays allo studio delle Leucemie Acute. Lo scopo principale della mia ricerca è stata la scoperta di nuovi biomarcatori e target terapeutici coinvolti nel processo leucemico mediante lo studio del profilo fosfoproteomico nei pazienti pediatrici affetti da LAL (LAL-T e LAL-B). Nel "Main Topic" veine descritto lo studio dei pazienti pediatrici affetti da LAL-T in cui si identifica PKCα come nuovo fattore prognostico di ricaduta. Questa kinasi è stata individuata mediante lo screening retrospettivo di 98 pazienti all'esordio di LAL-T, in cui 53 diverse proteine o fosfoproteine sono state analizzate con la tecnica degli RPPA. PKCα è risultata downregolata nel gruppo di pazienti LAL-T con maggiore incidenza di ricaduta. La minore attivazione di PKCα è risultata conseguenza di una bassa espressione genica di *PKCα*. Inoltre, inibendo PKCα in linee cellulari LAL-T mediante l'utilizzo di un inibitore commerciale di PKCα si è riscontrato un aumento della proliferazione. La metodica degli RPPA si è dimostrata essere un efficace approccio per la scoperta di molecole aberranti nelle vie di traduzione del segnale in cellule leucemiche. L'identificazione delle proteine coinvolte nei meccanismi tumorali mette in luce nuovi fattori prognostici: PKCα è risultata un promettente marcatore di rischio di ricaduta nelle LAL-T pediatriche. Lo studio dell'espressione di PKCα in una coorte allargata di pazienti LAL-T permetterà di confermare questo importante dato e di proporre PKCα come nuovo fattore prognostico nella stratificazione dei pazienti pediatrici affetti da LAL-T.

Nello studio delle LAL-B pediatriche, gli RPPA sono stati utilizzati per analizzare l' espressione/attivazione di 92 proteine/fosfoproteine in 118 pazienti affetti da LAL-B. Correlando le informazioni sulle vie di trasduzione attivate con la risposta alla terapia e con le caratteristiche genetiche dei pazienti, è stato possibile identificare nuovi biomarcatori che, una volta validati in un gruppo allargato di campioni LAL-B, potranno permettere una migliore stratificazione dei

pazienti e potranno essere usati come nuovi target terapeuti per lo sviluppo di cure più efficienti.

In particolare, nei pazienti a prognosi infausta si è osservato un aumento nell'attivazione/espressione di alcune vie di trasduzione del segnale coinvolte nella proliferazione cellulare. In primo luogo, i pazienti con traslocazione gel gene MLL presentano l'iperfosforialzione di BCL-2 come conseguenza dell'attivaizone di AMPK, suggerendo un ruolo di AMPK nell'inibizione dell'apoptosi. AMPK potrebbe dunque essere un nuovo potenziale target terapeutico. Il secondo risultato ha evidenziato una up-modulazione dell'attività di LCK nei pazienti con scarsa risposta al trattamento con Prednisone. Questa tirosina-kinasi potrebbe essere down-modulata mediante l'utilizzo di inibitori usati in clinica. Studi ulteriori approfondiranno questo dato al fine di identificare una terapia più adatta ai pazienti resistenti al Prednisone. Da questo studio è inoltre emersa un'associazione tra gli alti livelli di CICLINA E e l'incidenza di ricaduta. La CICLINA E è inoltre risutata maggiormente espressa nei pazienti ricaduti precocemente, generalmente associati a una prognosi infausta. E' stato successivamente approfondito il significato funzionale dell'attivazione di AMPK nei pazienti MLL riarrangiati ed il pathway di AMPK è risultato coinvolto in modo diretto alla sopravvivenza delle cellule LAL-B riarrangiate per MLL. Tale dato suggerisce AMPK come nuovo target terapeutico per la cura di questo tipo di leucemia.

L'affidabilità degli RPPA nell'identificare nuovi target e biomarcatori è stata riscontrata inoltre nell'ambito di progetti sviluppati in collaborazione con gruppi di ricerca nazionali e internazionali, tra cui lo studio delle Leucemie Mieloidi Acute nell'adulto.

Nel mio corso di dottorato ho potuto riscontrare che il metodo dei Reverse Phase Protein Arrays è una tecnologia sensibile, affidabile e high throughput mediante la quale è possibile identificare nuove molecole aberranti coinvolte nelle patologie ematopoietiche. Questi risultati potranno portare alla scoperta di nuovi marcatori prognostici e di target terapeutici per lo sviluppo di cure più specifiche ed efficienti.

AIM OF THE STUDY

Acute Lymphoblastic Leukemia (ALL) is an aggressive malignancy of lymphopoietic cells characterized by a clonal proliferation of blast cells originated from lymphoid precursors arrested at early stages of differentiation. ALL is the most common pediatric cancer, accounting for a quarter of all malignancies among children aged less than 15 years. This potentially catastrophic disease was once fatal in majority of patients but nowadays the introduction of risk-directed treatment and intensified cure protocols have improved the outcome of ALL pediatric patients. Indeed, current therapies achieve five-year event-free survival rate of about 80% in children receiving protocol-based therapy in the developed countries. Advances in genomics and molecular analysis of tumours have improved the ability to select patients that are likely to respond to a particular therapeutic agent, but substantial numbers of patients still experience relapse and have poor outcomes, failing to ever achieve a complete remission.

In order to develop new additional therapeutic supports and to assemble specific and personalized therapies for ALL pediatric patients, improving drug response and reducing drug toxicity, it is extremely important to identify novel therapeutic targets and new prognostic biomarkers on leukemic cells.

The aim of this research is to discover new candidate biomarkers and therapeutic targets involved in the leukemic process through the study of phosphoproteomic profiles of ALL pediatric patients (T-ALL and BCP-ALL), investigating the aberrant activation or expression of proteins involved in altered signal transductions pathways of leukemic cells by means of the Reverse Phase Protein Arrays (RPPA) technique.

The RPPA approach provides new information regarding malignant cells biology through the study of protein networks activation state, analyzing a wide

number of specimens using limited amounts of patients lysates. This high throughput method is useful to enhance understanding of the molecular mechanisms involved in leukemogenesis and the leukemic cells proliferative advantage.

In this context, during my PhD research, I applied the RPPA technique to the study of pediatric patients affected by ALL. The “Main Topic” of my thesis describes new data arisen from a phosphoproteomic study of T-ALL pediatric patients at diagnosis. The “Secondary Topics” report relevant results of BCP-ALL phosphoproteomic profiling, and show my participation in collaborative national and international research projects in the framework of the Phosphoproteomic group.

INTRODUCTION

1.1 ACUTE LYMPHOBLASTIC LEUKEMIA

Acute Lymphoblastic Leukemia (ALL) is the prevalent type of pediatric cancer, as well as the most common form of leukemia in children [1]. This lymphoid malignancy is characterized by the proliferation of lymphopoietic blast cells and represents a heterogeneous group of diseases with different morphological, cytogenetic, and immunologic features of the transformed cells [2][3]. The malignant clones in patients with ALL originate from normal lymphoid progenitor cells arrested at early stages of B- or T-lymphocyte ontogeny. About 85% of pediatric patients express B-lineage-associated antigens while approximately 15% of patients express the T-lineage-associated antigens [4][5][6]. Blast cells arrested at a specific premature stage of differentiation present an uncontrolled clonal proliferation and some genetic aberrations that bring to a pathological phenotype.

Cells implicated in ALL have clonal rearrangements in their immunoglobulin or T-cell receptor genes and express antigen-receptor molecules and other differentiation-linked cell-surface glycoproteins that largely recapitulate those of immature lymphoid progenitor cells within the early stage of normal T and B lymphocytes [7][8][9].

Steady progresses in the development of treatments, mainly consisting of multi-agent combination chemotherapy, has led to a cure rate of more than 80% in children with ALL (5-year event free survival), but about every fifth patient experiences resistance to treatment and relapses [4].

The precise pathogenetic events leading to development of ALL are still largely unknown. Only a few cases (<5%) are associated with inherited, predisponsing genetic syndromes, such as Down’s Syndrome, Bloom’s

Syndrome, ataxia-telangiectasia and Nijmegen breakage Syndrome, or with ionizing radiation and specific chemotherapeutic drugs exposure [5]. Retrospective identification of leukemia-specific fusion genes, hyperdiploidy, clonotyping rearrangements or immunoglobulin or T-cell-receptor loci in archived neonatal blood spots and studies of leukemia in monozygotic twins indicate clearly a prenatal origin in the majority childhood leukemias [6][10][11][12].

Chromosomal translocations that activate specific genes are a defining characteristic of human leukemia and, in particular, of ALL. Gene-expression patterns studied in large series of newly diagnosed leukemia have substantiated the idea that specific chromosomal translocation identify unique subtypes of the disease [13][14][15][16]. Translocations could activate transcription-factor genes involved in cell differentiation control, cell development or encoding proteins that play key roles in transcriptional cascades.

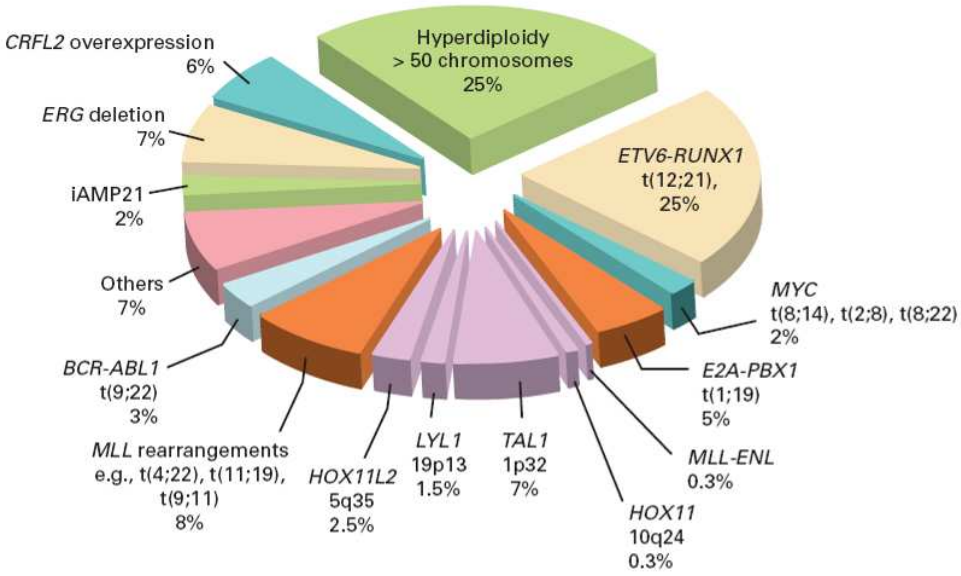

Fig. 1.1: Estimated frequency of genetic abnormalities in ALL. Violet areas refer to T ALL abnormalities, other colours indicate B ALL abnormalities (Modified from Pui et al., 2011 [4])

1.1.1 T-lineage Acute Lymphoblastic Leukemia (T-ALL)

In T-ALL the malignant clone derives from T-progenitor cells and express immature immunophenotypic markers characteristic of the T-cell lineage. T-ALL

is typically associated with very high white cell counts, mediastinal masses with pleural effusion and increased risk of leptomeningeal infiltration at diagnosis [7].

Flow Cytometry allows the identification of the different T-ALL phenotypes according to the markers expressed on T leukemic cells (see Table 1.1).

Table 1.1: T-ALL Immunophenotyping characteristics. (s= on the surface; cy= cytoplasmatic).

Early T	CD7+, cyCD3+, sCD3-, CD4-, CD8-, CD1a-
Intermediate T	cyCD3+, sCD3-, CD4+, CD8+, CD1a+
Mature T	sCD3+, CD1a-, CD4+ o CD8+

Of note, the most undifferentiated T-ALL disease subset is characterized by transformed Early T-cell Precursors (ETP) migrating from the bone marrow to the thymus and presents a distinct gene expression profiling, an immature immunophenotype (CD8-, CD1a-, CD5 weak) and prominent stem-cell features (stem-cell marker and myeloid marker coexpression). ETP-ALL is highly aggressive, resistant to conventional chemotherapy and associated with a poor prognosis [17].

T-ALL is a genetically heterogeneous disease in which several chromosomal and genetic alterations cooperate to promote the aberrant proliferation and survival of leukemic lymphoblasts. Actually, leukemia transformation of immature thymocytes is caused by a multistep oncogenic process and it involves numerous genetic abnormalities on oncogenes or tumor suppressor genes that bring to an uncontrolled cell growth. A variety of genetic events affecting cellular processes, such as differentiation, survival and cell cycle have been identified in T-ALL and results in differentiation arrest in nearly all stages of T-cell maturation in the thymus. In normal T-cell development, lymphoid progenitor cells migrate from the bone marrow towards the thymus where common $CD4^-$ $CD8^-$ double negative progenitor cells mature in T-cell receptor (TCR) $\alpha\beta$ or $\gamma\delta$ positive T-cells [18].

The alteration in the rearrangement process of TCR genes, identified in about 40% of T-ALL, leads to the activation of many genes involved in chromosomal translocations. Besides, other non-TCR gene mediated translocations could produce fusion products. Moreover, several genes become activated or inactivated due to the presence of specific point- or insertion/deletion mutations, or they could be affected by somatic copy number variations (amplifications and/or deletions) [19].

Among genetic alteration at diagnosis in childhood T-ALL, several translocations of different oncogenes (TLX1, TLX3, TAL1, LMO1, LMO2, HOXA) with TCR genes have an incidence of 40-50%. In particular, the TLX1 overexpression coming from t(10;14)(q24;q11) translocation is usually associated with a good outcome. By contrast, overexpression of TLX3 resulting from t(5;14)(q35;q32) translocation is associated with a high risk of poor outcome [20][21].

In 20-30% of T-ALL, an overexpression of TAL1 could be caused by a submicroscopic short-arm deletion of chromosome 1(1p32) and it could result in the SIL-TAL1 fusion, placing the protein coding domains of TAL1 under the control of the promoter region of SIL. Also the aberrant juxtaposition of TAL1 genes near TCR enhancers may result in its ectopic expression (about 40% of T-ALL). TAL1 activation could also results from the t(1;14)(p32;q11) or the variant t(1;7)(p32;q35). These aberrations characterize the most mature T-ALL subset committed to the $\alpha\beta$ lineage and they usually present a good treatment response [20][21].

Translocations that result in the formation of chimeric genes are less common in T-lineage than in B-lineage ALL. 5-10% of patients presents the translocation t(10;11)(p12;p14) with PICALM-MLLT10 fusion, typically associated with an adverse prognosis. A small subset of T-ALL patients (5-10%) presents MLL rearrangements, in particular T-ALL of TCR $\gamma\delta$ lineage, but their prognostic significance is not well defined. Another rare fusion gene is NUP214-ABL1, from the episomal amplification of 9p34, that leads to an abnormal tyrosine kinase activity [22]. In this region another rare deletion was identified, the

del(9)(q34.11q34.13), resulting in a conserved SET-NUP214 fusion product. Patients with PICALM-MLLT10, MLL rearrangements (<1%), inversion 7 (3%) and SET-NUP214 share a similar expression profile that is characterized by activation of HOXA genes [17][18].

The t(6;7)(q23;q34) was recently identified as a novel recurrent translocation in T-ALL that result in the activation of the MYB oncogene through the rearrangement with TCRβ: this MYB translocation was predominantly identified in very young children and is characterized by a peculiar gene expression profile [23].

Activating NOTCH1 mutations are present in more than 50% of T-ALL patients [24]. NOTCH1, whose gene is localized on chromosome 9, is a transmembrane receptor normally involved in correct stem-cell and thymocytes development. In fact, NOTCH1 signaling pathway is responsible for cell fate specification, which allows to thymocytes the differentiation into αβ or γδ T-cell lineage, and it is involved in the development of T-cell progenitors. Initially, a rare translocation of NOTCH1 with TCRβ gene, t(7;9)(q34;q34.3), has been described in 1% of T-ALL, resulting in a truncated aberrant NOTCH1 constitutively active form [25]. However, it was only after the identification of activating mutations in NOTCH1 in over 50% of T-ALL that the central role of NOTCH1 in the pathogenesis of this disease was fully appreciated. NOTCH1 mutations could affected its heterodimerization domain or the C-terminal PEST domain [24]. In a small group of patients, aberrant NOTCH1 activation results from inactivating mutations in the FBXW7 gene that encodes for a protein involved in NOTCH1 degradation [26]. Initial findings suggested an association between aberrant NOTCH1 signaling and improved treatment outcome but further investigations showed no prognostic association with NOTCH1 mutations [27]. Subclonal duplications of the chromosomal region 9p34 that include NOTCH1 are present in about 30% of pediatric T-ALL patients [28]. Although there is no clear relationship between the presence of NOTCH1 mutations and this 9p34 abnormality, duplication of this genomic region could result in changes of

NOTCH1 expression levels and contribute to NOTCH1 signaling activation in T-ALL.

60-70% of T-ALL patients also present a deletion of the chromosomal band 9p21, which contains the CDKN2A-2B tumor suppressor genes and it contributes to leukemia progress by loss of cell proliferation control [21][29]. These deletions affect the cell-cycle regulation but their presence still has unknown prognostic significance.

Several studies have described mutations of oncogene IL7R in 9% of T-ALL (gain-of-function exon 6 mutations) [30]. Recently, Zenatti PP et al. [31] showed that oncogene IL7R promotes tumor formation and cell transformation, activating the JAK-STAT signaling pathway. T-ALL patients were categorized into several oncogenetic subgroups, characterized by rearrangements and aberrant expression of transcription factors, such as TAL1, LMO1, LMO2, TXL1, TXL, HOXA. IL7R mutations induced a gene expression profile partially resembling that provoked by IL-7 and were enriched in the T-ALL subgroup comprising TLX3 rearranged and HOXA deregulated cases. Therefore, IL7R mutations were especially associated with the TLX and HOXA subgroups. No correlation was shown between mutated IL7R and specific T-ALL maturation stages and with NOTCH1 and/or FBXW7 mutations. Moreover, mutated IL7R did no associate with Prednisone response and survival. Authors proposed specific targeting of IL7R-mediated signaling as a treatment option for T-ALL. The attempt of the study was to investigate the correlation between IL7R mutations and the most important genetic aberrations and clinical/biological factors already describe in T-ALL pathogenesis with prognostic relevance.

T-ALL genetic features has been widely studied and much information describes the aberrant events arisen in T leukemic cells but association with disease prognosis is often not clear. Nowadays, the introduction of intensified treatment protocols has improved the outcome of T-ALL: current therapies achieve five-years-relapsed-free survival rates of about 75% in children but about 25% of patients remain at risk of early relapse [32]. In the light of current information on T-ALL disease, an integration between molecular characteristcs, prognostic

markers and candidated therapeutic targets is needed in order to achieve comprehention of T leukemic cell biology, improve the prognosis prediction and develop effective targeted therapies. Further advances require an in-depth understanding of T-ALL molecular genetics and leukemogenic pathways in order to identify new molecular players, such as prognostic biomarkers to improve T-ALL patients stratification and therapeutic targets to develop new therapeutic approches, more specific and less toxic.

Table 1.2: Characteristics and clinical outcomes of selected subtypes of childhood T-ALL. (Modified from Pui et al., 2011 [4])

Subtype	Frequency (%)	Clinical Implication	Estimated 5-Year Event-Free Survival (%)
T-cell			
TAL/LMO rearrangement	15-30	Good prognosis in some studies; potentially responsive to histone deacetylase inhibitor	?
HOX11 rearrangement	7-8	Good prognosis	?
HOX11L2 (*TLX3*) rearrangement	20-24	Poor prognosis in some studies	?
HOXA rearrangement	4-5	Poor prognosis; potentially sensitive to histone H3K79 methyltransferases inhibitor	?
NUP214-ABL1	6	Sensitive to tyrosine kinase inhibitor	~50 (survival)
MLL-ENL	2-3	Favorable prognosis	80-90
Early T-cell precursor	12	Poor prognosis; expressed myeloid or stem-cell markers	30-35
Cooperation mutations			
T-cell			
NOTCH/FBXW7 mutations	50	Favorable prognosis; potentially responsive to *NOTCH* inhibitor	90
PTEN-P13K-AKT pathway	~50	? Poor prognosis	?
CDKN2A/2B deletions	~70	? Potentially responsive to DNA methyltransferases inhibitor	?

Abbreviation: ALL, acute lymphoblastic leukemia.

1.2 PROTEIN PHOSPHORYLATION IN CELLS

The intracellular signaling network delicately regulates cellular homeostasis. This intracellular balance is carefully maintained by constant regulation of proteins post-translational modifications through the activity of a series of kinases, phosphatases, deacetylases and proteolitic enzymes.

Early in the 19th century it was known that phosphates could be bound to proteins; example of phosphoproteins were found in milk (caseins) and egg yolk (phosvitin) and at the time they were considered just a biological method to provide phosphorous as a nutrient. Phosphoproteins were considered a consequence of metabolic reactions not until the fifties, when phosphorylation emerged as key mechanism of cellular life regulation. In 1954 phosphate transfer was observed from an enzyme onto another protein and this biological reaction

was called *phosphorylation*. A year later Fisher and Krebs showed that an enzyme involved in glycogen metabolism was regulated by addition and removal of a phosphate and this suggested that enzyme activity could be control by reversible phosphorilation [33][34][35]. Today, it is known that protein phosphorylation plays a key role in signal transduction pathways in all eukaryotic cells, acting as the principal molecular mechanism of protein activity regulation. Protein phosphorylation is a ubiquitous and reversible post-transcriptional modification which affects the activity of about one third of all proteins in a cell [36]. Phosphorylation and dephosphorylation play an important role in regulation of enzymatic activity, protein conformational changes, protein-protein interaction, protein function and cellular localization [37][38].

Many human diseases have been recognized to be associated with *abnormal phosphorylation* of cellular proteins: alteration of signalling pathways can leads to an unbalance of cell metabolism, proliferation, survival and differentiation that characterize pathological cells such as cancer cells. The study of altered signaling pathways of phosphoproteins offers a real opportunity to identify aberrant molecules to propose as prognostic markers or as novel therapeutic targets for the development of new treatments.

1.3 PROTEIN ARRAYS

To investigate and understand the molecular basis of cancer the integration of distinct but complementary knowledge from the fields of genomics and proteomics is required [36][39]. Genomics and trascriptomics provide information on potential genetic defects that may cause disruptions in cell signalling pathways. Proteomics, on the other hand, focus on crucial details about the functional state of these disrupted pathways. This molecular integration provides a complete view of defective cellular machinery governing pathological states, in order to reveal potential drug targets and diagnostics molecules for disease prognosis or treatment [40][41][42][43].

The dynamic nature of proteome allows to closely monitor changes in the state of a cell, tissue or organism over time and to follow the course of a disease and track its pathogenetic mechanisms, as well as the response to therapy.

Protein microarrays provide a powerful and high throughput tool for profiling and comparing protein pathways in order to identify novel diagnostic and prognostic biomarkers for cancer and other diseases. They also provide critical information about protein post translational modifications, such as the phosphorylation states of proteins, which reflect the activation state of signalling pathways and networks. Protein microarray technologies provide the unique opportunity to profile tissues, assess the activity of signalling pathways within isolated cell populations and characterize “circuit maps” of cellular signalling pathways in normal and diseased cells. The identification of critical nodes or interactions within the network is a potential starting point for drug development and/or design of individual therapy regimens [44][45].

Currently there are two main protein arrays approaches able to generate protein network information (Fig. 1.2), the *Forward Phase Protein Arrays* and the *Reverse Phase Protein Arrays*. The latter is the one adopted in the studies reported in this thesis.

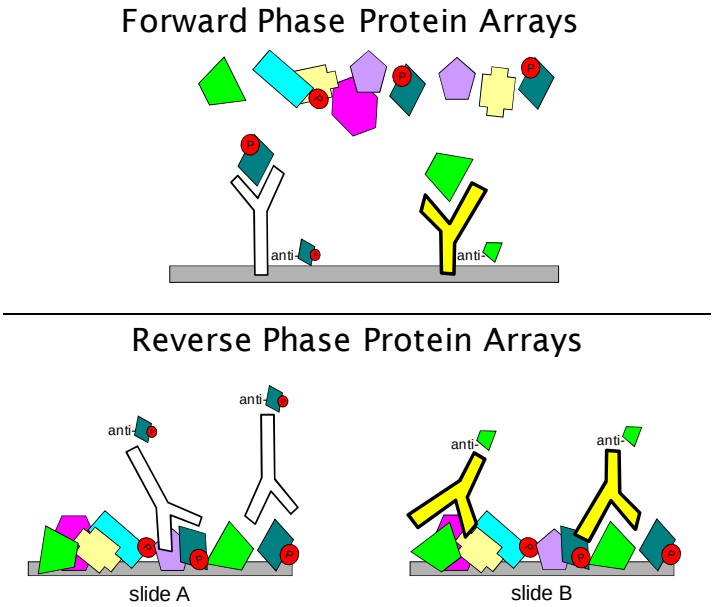

Fig. 1.2: Forward and Reverse Phase Protein Arrays.

1.3.1 Forward Phase Protein Arrays

In the first format, called Forward Phase Protein Arrays, a labelled bait molecule is immobilized on the substratum. Multiple antibodies can be immobilized on each array. Each spot represents one bait molecule. The array is incubated with one test sample that contains several different analytes of interest, as depicted in Fig. 1.2. The captured analytes can be detected by labelling the analyte of interest directly, typically with a fluorescent molecule, or with a second tagged antibody [46][47][48]. In this last case, named sandwich array, one antibody is required to bind the analyte of interest to the substratum and a second antibody binds a different epitope on the same molecule [49][50]. The forward phase array permits the simultaneous analysis of multiple analytes present in one sample.

1.3.2 Reverse Phase Protein Arrays

In the Reverse Phase Protein Arrays (RPPA) format, protein microarray immobilizes the protein to be analyzed. Each spot represents an individual test sample, allowing an array to be comprised of multiple, different samples. Each RPPA slide is probed with a single detection molecule (one slide = one antibody) and a single analyte is concurrently measured across multiple samples (Fig. 1.2). This format allows multiple samples to be analyzed under the same experimental conditions for any given analyte (same amount of time together with same concentration of primary antibody, secondary antibody, and amplification reagents). The adoption of the same experimental conditions facilitates the comparisons between samples since experimental variability is eliminated.

The benefit of RPPA is their high throughput capabilities using low sample volumes (for lymphocytes analysis we started from 5-10 x 10^6 cells). Typical RPPA are printed with 20-50uL of whole cell lysate and a single microarray can accommodate dozens to hundreds of samples. Printed slides may be stored and probed at a later date when additional proteins of interest are identified, or additional probes are developed.

These benefits are perfectly matched for molecular profiling of clinical patients samples because frequently only a small amount of patient material is available for molecular analyses. RPPA have a sensitivity of picograms per mL with clinical grade precision [51][52].

The biggest limitation of RPPA is that its success is highly dependent on the quality of the antibody set used: all the antibodies employed for the staining have to be extensively validated for single band specificity by Western Blot, to ensure its reactivity and its high specificity [53].

Thus, RPPA can measure the activation levels/phosphorylation of large numbers of signalling proteins at once from small clinical samples in a very reproducible, precise, sensitive and high-throughput manner [46][47][54].

In RPPA, protein lysates from cells are serially diluted with lysis buffer into four-point dilution curves and immobilized in small spots on nitrocellulose coated glass slides in duplicate. Each RPPA slide is then stained with a specific primary antibody, previously validated for a single band specificity by Western Blot. The primary antibody is detected with a biotynilated secondary antibody, next a streptavidin-biotin-peroxidase complex is allowed to bind to the secondary antibody and an amplification reagent amplifies the number of biotin molecules available for binding of the next reagent, streptavidin peroxidase. Antibody staining is revealed using DAB as chromogen substrate (see Fig. 1.3).The binding of each protein/phosphoprotein is quantified using the commercially available software Microvigene Software. This software is able to localize the spots, remove artefacts and subtract the background, calculating pixel intensity for each spot. Then the software subtracts the corresponding negative control slide, which is a slide stained with only the secondary antibody in order to subtract aspecific signal. The signal intensity is then normalized with the total protein content using a slide stained with the Fast Green FCF dye (Sigma). The data processing will generate a single value for each sample relative to each phosphorylated protein.

RPPA have been successfully applied to the analysis of signalling pathways in several cancers to provide clues regarding prognostic markers and the identification of new drug targets, i.e. prostate tumor [51][55][56], ovarian cancer [57][58][59][60], breast cancer [61][62][63][64][65][66][67][68], colorectal cancer [69] and childhood rabdomyosarcoma [70].

Also hematopoietic malignancies have been studied by means of RPPA. Phosphoproteomic analyses has been conducted in follicular lymphoma and highlighted new prognostic markers (Bcl2/Bak and Bcl2/Bax) and some clinically relavant molecules involved in prosurvival pathway signals [71][72]. Acute Myeloid Leukemia (AML) have been extensively studied by RPPA [52][73][74][75][76][77]. The main results identified several protein signatures correlated with relapsed, remission and overall survival. Moreover, new prognostic markers have been discovered. For example, high levels of FOXO3A has been associated to poor outcome in AML and proposed as potential therapeutically targetable prognostic factor. In pediatric BCP Acute Lymphoblastic Leukemia, an increased activation/expression of several pathways involved in cell proliferation in poor clinical prognosis patients was found. MLL-rearranged tumours revealed BCL-2 hyperphosphorylation through AMPK. Moreover, in patients with poor clinical response to Prednisone a LCK up-

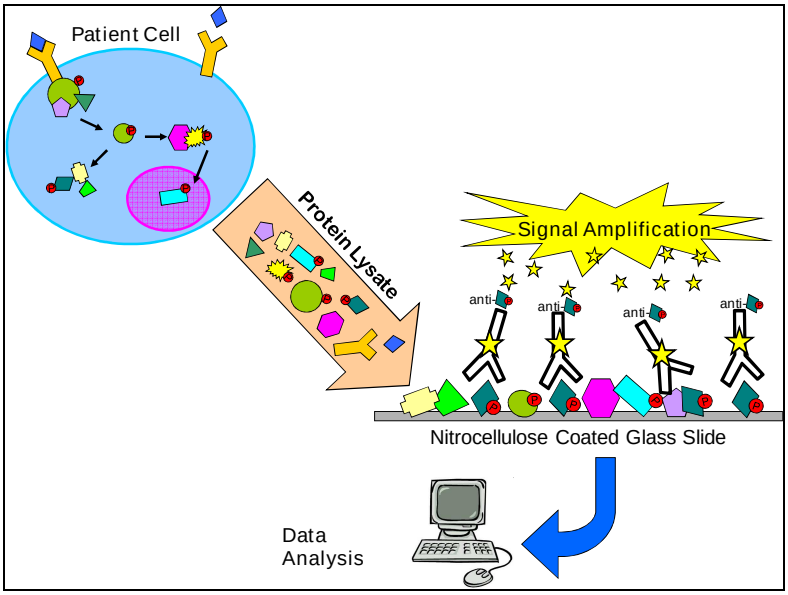

Fig. 1.3: RPPA experimental protocol.

modulation was observed. Further an association between high levels of CYCLIN E and relapse incidence was identified [78].

In studies described in following chapters, Reverse Phase Protein Arrays technique has been applied to the analysis of several hematopoietic diseases revealing its usefulness in identification of new prognostic markers and therapeutic targets.

MAIN TOPIC

"LACK OF PROTEIN KINASE C ALPHA IS ASSOCIATED WITH POOR PROGNOSIS IN PEDIATRIC T-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA"

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ORAL PRESENTATION at the 53rd Annual Meeting of the American Society of Hematology (San Diego, California - Dec. 10th-13th 2011)
BLOOD, Journal of the American Society of Hematology. Vol. 118, N0 21:p338. PUBLICATION NUMBER: 744

2.1 INTRODUCTION

2.1.1 Protein Kinases C

The discovery of Protein kinase C (PKC) as a calcium and phospholipid dependent serine/threonine-specific protein kinase dates back more than three decades. It was the first enzyme which was identified as a receptor for diacylglycerol, a lipid which is generated as a product of phospholipase Cγ (PLCγ) activation, a well known signal transducer downstream of many receptors, such as tyrosine kinase receptors [79]. Subsequently, a number of isoforms have been discovered that are involved in a wide variety of biological events within the cell such as cell growth, cell-cycle regulation, cellular survival, malignant transformation, apoptosis and differentiation. The PKC family, which consists of up to 10 members, is composed of a number of individual isoforms that belong to three distinct subfamilies by the virtue of the structural differences that dictate

their ability to respond to cofactors Ca^{2+} and diacylglycerol (DAG) [80]: the classical (or conventional) PKC isoforms α, βI, βII, γ; the novel PKC isoforms δ, ε, θ, η, μ, and the atypical PKC isoforms λ and ζ. All PKC subfamilies require negatively charged phospholipids like phosphatylserine for optimal activity. The classical PKC isoforms depend on from $Ca^{2+}$and DAG for their activation, the novel PKC isoforms are Ca^{2+}-indipendent but do require DAG, whereas the atypical subfamily members are unable to bind Ca^{2+} or DAG [80][81].

Structurally, PKC presents a N-terminal regulatory domain and a C-terminal catalytic domain linked by a V3 hinge region. The N-terminal regulatory domain contains an autoinhibitory pseudosubstrate domain, two membrane-targeting modules termed C1 (DAG binding) and C2 (Ca^{2+} binding) and several phosphatidylserine (PS) binding domains. Atypical PKCs contain an atypical C1 domain that can not bind DAG. The catalytic core, highly conserved between family members, contains a C3 (ATP-binding) and a C4 (substrate-binding) domain [82][83][84][88]. In Fig. 2.1 the structure of the PKC family proteins is depicted.

Most of the PKC isoforms are expressed ubiquitously and only some of them are restricted to specific organs or cell types, i.e. within the classical PKC

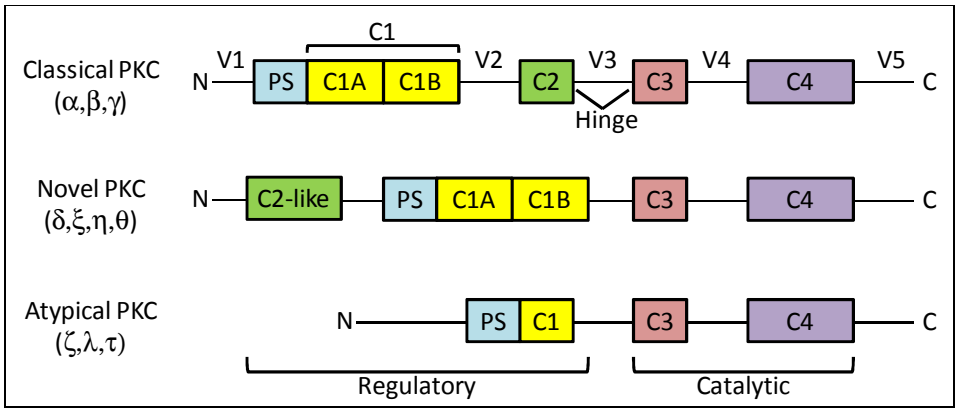

Fig. 2.1: Structure of the PKC family proteins. PKCs are grouped into three subclasses based on regulatory domain composition, which dictates the cofactor dependence. A regulatory domain comprises two basic modules, C1 and C2 domains. The C1 domain is a tandem repeat for cPKCs and nPKCs, but not for aPKCs. This domain mediates DAG/phorbol-ester-binding and contains autoinhibitory pseudosubstrate (PS) sequence in its N-terminus. C2 domain mediates Ca2+-regulated phospholipid binding in cPKCs. All kinases have a conserved kinase domain in the C-terminus: C3 domain is the ATP-binding domain and C4 domain is the substrate-binding kinase core. Catalytic domain activity is highly regulated by phosphorylation and co-factor binding. Modified from Michie AM, 2005 [86].

subfamily PKCγ has been shown to be specifically expressed in neuronal tissue, whereas PKCβ is preferentially expressed in pancreatic islets, monocytes and brain tissue [85].

Since different PKC isoforms have ubiquitous expression patterns, which results in a functional redundancy between the isoforms, define biological specific roles for each of them is a complex task. Moreover, PKC isoforms can be activated by a variety of stimuli ranging from members of the G protein-coupled receptors, tyrosine kinase receptors and non-receptor tyrosine kinases [85].

2.1.2 Protein Kinase C alpha - PKCα

PKCα, a protein of 672 amino acids, is ubiquitously expressed in many tissues and has been associated with a number of biological functions such as cell proliferation, differentiation, cell cycle control, apoptosis, cell survival, cell adhesion and cell motility. Cellular responses depend on temporal PKCα activation, on which downstream proteins are targeted and on tissue specificity [86][87].

2.1.2.1 Activation of PKCα: three mechanisms lead to the generation of a mature and catalytically-competent PKCα molecule

Phosphorylation. A series of phosphorylation events are essential to maturation of the nascent PKCα molecule to a catalytically competent one. The catalytic domain of PKCα is characterized by three conserved phosphorylation sites: the activation loop (Thr-497), turn motif (Thr-638), and hydrophobic C terminal motif (Ser-657). Phosphorylation at these sites is controlled by two tightly regulated mechanisms: the first, within the activation motif, is independent of internal PKCα activity, while the second is an autophosphorylation-mediated event at the turn motif and the hydrophobic motif [88][89][90]. In Fig. 2.2 the detailed structure of PKCα is depicted.

Phosphoinositide-dependent kinase-1 (PDK1) plays a crucial role in phosphorylating the activation loop site of cPKC isoforms, thus initiating their maturation. Phosphorylation at the activation loop promotes autophosphorylation

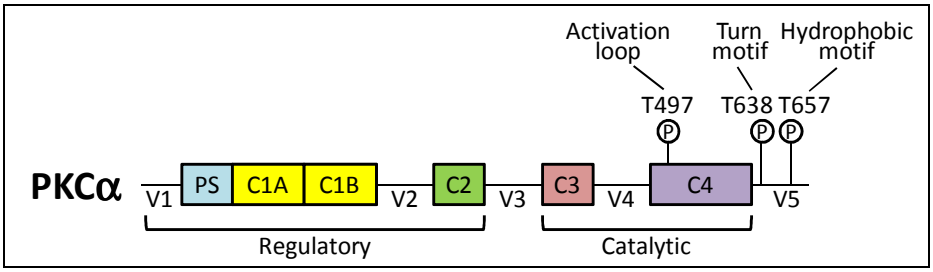

Fig. 2.2: Detailed structure of PKCα, a 671 amino acid protein. It is regulated by phosphorylation at the following sites: T497 in the activation loop; T638 in the turn motif; S657 in the hydrophobic motif. Modified from Michie AM, 2005 [86]

at the turn motif and the hydrophobic C terminal motif [91][92]. These serial phosphorylations result in a conformational change in the PKCα molecule, such that the pseudosubstrate sequence (see PS region in Fig. 2.1) blocks the active site, maintaining PKCα in a 'primed' but inactive state until stimulation of upstream signalling molecules activate the enzyme. Importantly, dephosphorylation of these sites is an essential mechanism to control PKC signalling capacity and is mediated by protein phosphatase 2A (PP2A) [93]. Phosphorylation at the turn motif stabilizes PKCα into a catalytically-competent, thermally stable, phosphatase resistant conformation. Hydrophobic motif phosphorylation plays a role in regulating the intracellular distribution of PKCα and contributes to its phosphatase-resistance and thermal stability. Phosphorylation of Ser 657 is currently used as a marker for the complete PKCα activation [94][95].

Cofactor requirement. In order to achieve the fully catalytically activation, phosphorylated PKCα in the cell cytoplasm requires additional regulatory mechanisms. The PKCα 'primed' state is maintained, at least in part, by the presence of an N-terminal autoinhibitory, pseudosubstrate sequence that occupies the active site within the catalytic domain rendering it unable to bind and phosphorylate substrates [80]. In addition also other regions of the regulatory domain contribute to the autoinhibition of PKCα activity [96].

The binding of specific receptors within the cell cause the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP2), by Phosphatidylinositol-specific

Phospholipase C (PLC), into the calcium mobilizer inositol-1,4,5-triphosphate (IP3) and the allosteric activator of novel/classical PKC DAG [80].

DAG is able to recruit PKC to the plasmatic membrane and increases its affinity for phosphatidylserine, an aminophospholipid localized exclusively in the cytoplasmic surface of the plasmatic membrane. Ca^{2+} also facilitates translocation, increasing the affinity of the C2 domains of cPKC for anionic membranes. Thus, cofactors binding to the C1 and C2 domains enable PKCα to bind to the plasmatic membrane and generate enough energy to allow a conformational change that facilitates the release of the pseudosubstrate region from the substrate-binding site [82]. Therefore cofactor interaction with PKCα plays an essential role in mediating its translocation from the cytosol to the plasmatic membrane and subsequent activation.

Intracellular localization. The intracellular localization of PKC isoforms is strictly regulated by several proteins at different stages of their maturation: A kinase anchoring protein (AKAP) family member docks phosphorylated but inactive PKCs close to their substrates [97]; receptor for activated C kinases (RACKs) anchors active phosphorylated PKCs at specific cellular locations close to their substrates [98]; proteins that interact with C kinases (PICKs) act as substrate for phosphorylation [99]. The association of PKCα with scaffolding proteins brings it into close proximity to downstream substrates.

PKCα and PKA colocalize with gravin/AKAP250 in human neurons. The association with gravin is increased upon inhibition of PKCα activity in neurons [100].

A 700 kDa phosphoprotein AHNAK/desmoyokin has also been shown to localise PKCα towards specific substrates. AHNAK acts as a scaffolding network for both PLCγ and PKCα, enabling a PKCα-mediated activation of PLCγ, which is only possible because these two enzymes are placed in close proximity by their ability to bind ANHAK [101].

Thus, PKCα is able to mediate a number of biological events in different cellular contexts, by virtue of tissue specific binding proteins.

2.1.2.2 PKCα biological processes

The activation of PKCα results in a variety of biological functions including cell proliferation, differentiation, cell cycle control, apoptosis/cell survival, cell adhesion and alteration in cell morphology. PP2A can negatively regulate PKCα activity, by deposphorylating the kinase (Fig. 2.3).

PKCα can act with opposite roles and cellular response induced by its activation or overexpression varies depending on the types and conditions of cells. In cancer, PKCα has been described as tumor suppressor as well as having an oncogenic role.

Proliferation and differentiation. PKC isoforms can act in tandem to execute biological processes. Many studies highlight the pivotal role that PKCα plays in mediating a number of signalling pathways and establish that PKCα plays a central role in the control of cellular proliferation and differentiation [102][103][104][105][106][107][108][109].

In cells overexpressing PKCα an increase in proliferation has been observed in several cell types, which is demostrated through the study of its downstream targets. Indeed PKCα has been shown to phosphorylate Raf-1 kinase, where it leads to the activation of the extracellular-signal regulated kinase-mitogen

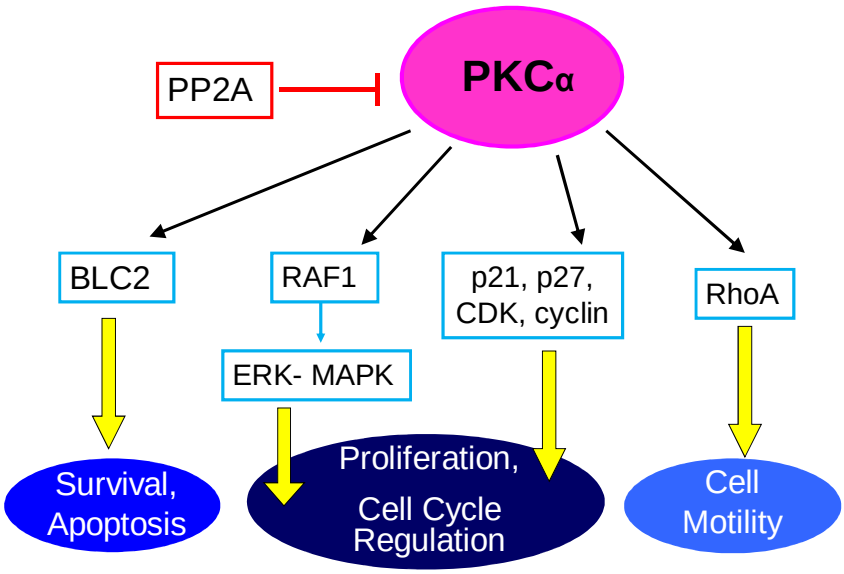

Fig. 2.3: Biological outcome of PKCα activation. Modified from Michie AM, 2005 [86]

activated protein kinase (ERK-MAPK) cascade, thus promoting cellular proliferation [105][106].

Cell cycle regulation. PKCα has the ability to act as a growth stimulator or inhibitor depending on the cell system. PKCα has been described as a promoter of cell cycle progression: expression of catalytically active PKCα constructs activate the cyclin D1 expression, via the AP-1 element of the cyclin D1 promoter, thus enhancing cell cycle progression. PKCα activation also promotes cell cycle progression in glioma cells through the upregulation of the cyclin–cyclin dependent kinase (CDK) inhibitor $p21^{waf1/cip1}$. Moreover, $p21^{waf1/cip1}$ can act as an assembly and activity-promoting factor for cyclin–CDK complexes, thus promoting cell cycle progression in certain cell types [110][111].

In contrast, PKCα can also play an inhibitory role in cell cycle regulation. In intestinal epithelial cells, PKCα-mediated signalling events result in a block of the cell cycle into the G_0 phase. This is due to a downregulation of cyclin D mediated by PKCα and to an increased expression of $p21^{waf1/cip1}$ and $p27^{kip1}$ which inhibit CDK complex activity, driving the cells to exit the cell cycle and enter the G_0 phase [112]. A similar observation was made in pancreatic cancer cells, where PKCα-mediated signalling inhibited cell growth by arresting the cells in the G_1 phase and inducing $p21^{waf1/cip1}$ expression with consequent inhibition of CDK2 activity and hypophosphorylation of Rb [113].

Cell survival/apoptosis. Since loss of PKCα activity is generally correlated with apoptosis induction, PKCα-mediated signals seem to act as survival promoters [114][115].

The dependence of cell survival on PKCα activity has been shown in many cell lines and different mechanisms could be involved. In salivary epithelial cells, the absence of PKCα leads to the activation of a PKCδ-caspase 3-dependent apoptotic pathway [116], whereas in COS cells the absence of PKCα activity results in a downregulation of Bcl-2 expression levels [114].

PKCα can mediate cell survival by increasing the phosphorylation/ expression levels of the anti-apoptotic proteins Bcl-2 [117][118][119] and/or Bcl-XL [115].

Indeed, PKCα can directly phosphorylate Bcl-2, a process that can be reversed by PP2A [117][118]. Evidences suggest that overexpression of PKCα suppresses mitochondrial PP2A activity, thus generating a supply of constitutively phosphorylated Bcl-2 at the mitochondria [118]. In support of this finding, PKCα and Bcl- 2 have been shown to colocalize at the mitochondrial membrane [117].

PKCα and cell adhesion. Cells with high PKCα activity display anchorage-independent growth, morphologic alterations and increased metastatic capacity, suggesting that PKCα plays a major role in the regulation of cell shape [104][120]. PKCα localises with and phosphorylates many proteins that are associated with cell migration and focal adhesion formation, including vinculin, syndecan-4, fascin and β1 integrin [121][122][123][124].

PKCα has significant effects on the regulation of cell migration as a result of its physical association with β1 integrin, which is responsible for promoting actin assembly. Activated PKCα leads to an upregulation of β1 integrin expression in mammary epithelial cells [121]. While the association of PKCα with β1 integrin occurs in the absence of the catalytic domain, PKCα activity is required for the induction of cellular migration. This may be achieved at least in part by PKCα–mediated activation of Rho, leading to Rho-dependent actin cytoskeletal rearrangement [125]. PKCα can also associate with the actin bundling protein fascin at the plasmatic membrane [124]. This association, in contrast to the study above, appears to decrease cell motility in skeletal myoblasts. Loss of fascin from actin-based structures during the initial stages of cell spreading occurs in a PKCα dependent manner [126], thus illustrating opposing roles of PKCα in mediating cell migration.

2.1.2.3 PKCα and cancer

PKCα has long been recognized to play a role in regulating aspects of tumor growth and development, although its involvement is complex and highly tissue-dependent. It can act as a tumor promoter or as tumor suppressor depending on the type of tumur [127].

Overexpression of PKCα has been demonstrated in tissue samples of prostate, endometrial, high grade urinary bladder and hepatocellular cancers [128][129][130][131][132][133], whereas for haematological malignancies up- and down-regulation of PKCα has been described [134]. Downregualtion of PKCα has been observed in basal cell and colon cancer [135][136][137].

A mixed picture also pertains to breast cancer cells, where PKCα has been extensively studied. Whereas activation or overexpression of PKCα has been shown in breast cancer cells and breast tumor samples [138][139], a downregulation has also been demonstrated [140].

Due to its implication in a larger number of cancers, PKCα has become a target for therapies such as the use of antisense oligonucleotides (ASO) that target the 3'-UTR untranslated region of human PKCα mRNA, short interfering RNA (siRNA) or PKC inhibitors directed specifically against PKCα [141][142][143].

Many studies indicate a significant therapeutic value for specific targeting of PKCα expression. Interestingly, activation of PDK1 in mammary epithelial cells can lead to tumorigenesis and cells exhibited anchorage independent growth due to an overexpression of PKCα [120]. Complementary studies carried out in a number of human cancer cells revealed that the reduction of PKCα expression levels, utilising ASO or siRNA techniques, reduced the proliferation rate of these cells [141][143]. Additionally, the expression levels of the oncogenes *H-RAS*, *JUN* and *FOS* were also reduced in these cancer cells, possibly due to a decrease in AP-1 activity [141].

PKCα appears to have opposing effects on the cell cycle regulation depending on the cell system studied. Indeed, PKCα activation can promote the progression of cells towards the S phase, increasing proliferation, or enable the cells to withdraw into the G0/G1 phase [111][112]. In chronic lymphocytic leukaemia (CLL) a progressive accumulation of long-lived mature B cells is observed due to a reduction in apoptosis and a cell cycle arrest in the G0/G1 phase [144]. The underlying apoptosis defect in B-CLL has been attributed to both higher levels of the anti-apoptotic Bcl-2 family member proteins and mutation of the p53 tumor

suppressor gene [144]. Due to the increased levels of apoptosis induced by cPKC inhibitors, it has been suggested that cPKCs may mediate survival signals in B-CLL cells [142][145]. Some studies suggest that PKCα regulates the phosphorylation and expression levels of Bcl-2, rendering the cells less sensitive to apoptosis [117][118][119]. Therefore, reducing PKCα expression levels in B-CLL cells may increase the rate of apoptosis. In support of this, treatment of Bcl-2 overexpressing mammary adenocarcinoma cells with PKC inhibitors against PKCα in combination with anticancer drugs counteracted the cytoprotection offered by Bcl-2. This correlated with an inhibition of cell cycle progression and also inhibited anchorage independent tumour cell growth [146].

The role of PKCα in Acute Myeloid Leukemia (AML) has been associated with differentiation, apoptosis and cell adhesion. PKCα was differentially expressed in patients with AML compared to patients with acute lymphoblastic leukemia and CLL [147]. PKCα and Bcl2 expression were measured in peripheral blood mononuclear cell lysate from AML patients. The concomitant expression of PKCα and Bcl2 resulted in an independent prediction of poor survival compared to classical cytogenetics and both proteins were highly expressed in particular in M4 AML patients [148]. Studies in AML cell lines using different PKCα activators (retinoic acid, vitamin D3, leukotriene D4, irradiation and camptothecin) described the downstream effects: PKCα activation is associated with increase in differentiation, apoptosis (with increase of phospholipase D, lamin B and annexin I) and cell adhesion (activation of Raf1, MAPKinases and integrins). On the other hand, PKCα activation is associated with increased immunomodulation (activation of Raf1, MAPKinases, AP1, NFκB, TNF-α and interleukein1) and also with a reduction of tumor cell apoptosis through Bcl2 activation [149][150][151].

2.2 AIM OF THE STUDY – MAIN TOPIC –

T-ALL accounts for about 10-15% of pediatric leukemias. Although the prognosis of T-ALL pediatric patients has improved in recent years thanks to intensified treatments, about 25% of T-ALL patients remain at high risk of relapse. It is therefore very important to identify prognostic biomarkes and new molecular targets in order to improve T-ALL patients' stratification and suggest new more specific and less toxic therapeutic approaches. T-ALL genetic features have been widely studied and a lot of literature describes specific aberrant genomic events of T leukemic cells, but associations between molecular aberrations and prognosis is not often clear.

The aim of this study is the identification of aberrantly activated or expressed proteins in pediatric T-ALL patients at diagnosis by means of the Reverse Phase Protein Array (RPPA) technique. RPPA approach can provide new information regarding malignant cells biology through the study of protein networks activation state. This innovative technique can measure the activation levels/expression of large numbers of signalling proteins from small clinical samples in a very reproducible, precise, sensitive and high-throughput manner. Correlation of protein expression/activation with clinical response and genetic information will enable us to identify new protein biomarkers that could be used for patient stratification and targeted therapy trials.

2.3 MATERIALS AND METHODS

2.3.1 Patients

Bone marrow samples from 98 T-ALL pediatric patients at diagnosis were retrospectively studied. Diagnosis was made according to standard cytomorphology, cytochemistry and immunophenotypic criteria [152]. Patients studied were enrolled in AIEOP treatment protocols. Permission for this study was obtained following the tenets of the Declaration of Helsinki.

Samples were collected at the Pediatric Oncohematology Laboratory of Padova (Italy) between 1990 and 2006 and stored in the BioBank in liquid nitrogen in FBS+DMSO. Mononuclear cells from patients bone marrow were separated by Ficoll-Hypac technique (Pharmacia, Uppsala, Sweden). Samples selected had a blood blast percentage between 70% and 98%. Patients molecular and clinical features were collected and resumed in Table A 2.1 in chapter 2.6-Appendix.

2.3.2 T Cell Lines

Human T leukemia cell lines were purchased from DMSZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). CCRF-CEM, DND41, MOLT3, TALL1, P12ICHIKAWA cell lines were cultured in RPMI 1640 (Biochrom AG, Berlin, Germany) with 10% or 20% FBS, penicillin (100U/ml)(GIBCO, Invitrogen Life Technologies, Carlsbad, CA) and streptomycin (100μg/ml)(GIBCO) and maintained at 37°C in a humidified atmosphere with 5% CO_2.

2.3.3 T cells from healthy donors

Mononuclear cells from peripheral blood (PB) of 4 healthy donors were isolated using Ficoll-Hypaque (Amersham Biosciences) density gradient centrifugation. T cells from healthy donors were isolated from peripheral blood mononuclear cells by depletion of non T-cells using human Pan T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). A Miltenyi autoMACS magnetic cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to

purify T cells and then isolated cells purity was confirmed by flow cytometry. The purity of the isolated T cells was 99%, as determined by flow cytometric analysis. These T cells were used as the normal control (calibrator) in RQ-PCR experiments.

2.3.4 RPPA

Cell Lysis. Frozen samples cells were thawed on ice and then washed with ice-cold PBS 1X. Cells were then resuspended in an appropriated cold lysis buffer on ice for 20 minutes. Lysis buffer: TPER reagent (Pierce, Rockford, IL), 300mM NaCl, 1mM Na orthovanadate, 200mM PEFABLOC (AEBSF) (Roche, Basel, Switzerland), 1μg/mL Aprotinin (Sigma-Aldrich, St. Louis, MO), 5mg/mL Pepstatin A(Sigma), 1 mg/mL Leupeptin (Sigma). Cell lysates were then cleared by centrifugation and supernatants were collected and stored at -80°C until use.

Protein Quantification, Samples Dilution And Boiling. Proteins were then assayed for protein concentration by BCA-Protein Assay Kit (Pierce). Cell lysates were then diluted to 1mg/mL in a mixture of 2X Tris-Glycine SDS Sample Buffer (Invitrogen Life Technologies) plus 5% of B Mercaptoethanol. Lysates were boiled for 8 minutes and stored at -80°C.

RPPA printing. Protein lysates were loaded into a 384-well plate and serially diluted with dilution buffer into four-point dilution curves (from undiluted to 1:8). Three commercial cell line lysates (Jurkat, HeLa+ Pervanadate, Reh+Doxorubicin) (BD Biosciences, Franklin Lakes, NJ) were used as positive controls for antibody staining. Samples were printed in duplicate, in a 4 points dilution curve, on nitrocellulose-coated glass slides (FAST slides, Whaterman Schleicher & Schuell, Florham Park, NJ) using the 2470 Arrayer (Aushon BioSystems, Burlington, MA) (see Fig. 2.4). Printed slides were stored desiccated (Drierite, Sigma) at -20°C until use.

RPPA staining. Selected slides were stained with FAST GREEN FCF (Sigma) 0.01%, a fluorescent dye used to estimate the total protein amount for each printed sample. Fast Green FCF stained slides were scanned by ScanArray 4000 (Packard Biochip Technologies, Billerica, MA).

Fig. 2.4: Aushon 2470 Arrayer. The *Aushon Arrayer 2470* uses the *solid pins* technology.

Before antibody staining, slides were blocked in blocking solution (I-Block 2% - Applied BioSystems, Foster City, CA-, 0.1% Tween20 - Sigma - in PBS 1X) for at least 3h at room temperature. Then, blocked slides were stained with antibodies on an automatic slide stainer (Dako Autostaines Plus, Dako Cytomation, Carpinteria, CA) using the CSA kit (Dako Cytomation).

Each RPPA slide is stained with a specific primary antibody. Proteins on the slides are recognized by the primary antibody, which is subsequently detected by a biotinylated secondary antibody. Afterwards, a streptavidin-biotin-peroxidase complex (SABC) binds to biotinylated secondary antibody. An amplification reagent amplifies the number of biotin molecules available for binding of the next reagent, streptavidin peroxidase. Antibody staining is revealed using the diaminobenzidine (DAB) as chromogen substrate. Peroxidase cuts the DAB to originate a brown precipitate. This method allows a great signal amplification. After staining, slides were air dried and scanned at 600dpi (Scanner EPSON Perferction V350 Photo).

An example of RPPA stained slide is reported in Fig. 2.5.

In this study, 53 antibodies able to key signalling molecules involved in different cell pathways such as proliferation, survival, growth and apoptosis were used. See Table A 2.2 in chapter 2.6-Appendix.

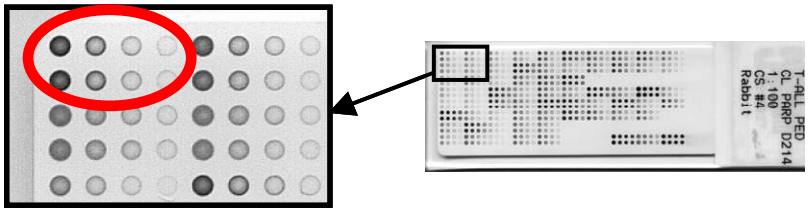

Fig. 2.5: Example of RPPA stained slide. Each patient lysate was printed in a four-point dilution curve ranging from undiluted to 1:8 in duplicate (see red selection in the image).

2.3.4.1 Antibody validation for RPPA staining

Each primary antibody to be used in RPPA was previously subjected to an extensive validation for single band specificity by Western Blot, using specific cell lysates as positive controls. Successful detection of proteins is highly dependent on the quality of the antibodies: it is therefore crucial to ensure the sensitivity and specificity of each primary antibody. In Fig. 2.6 and Fig. 2.7 examples of antibodies validations by Western Blot are reported.

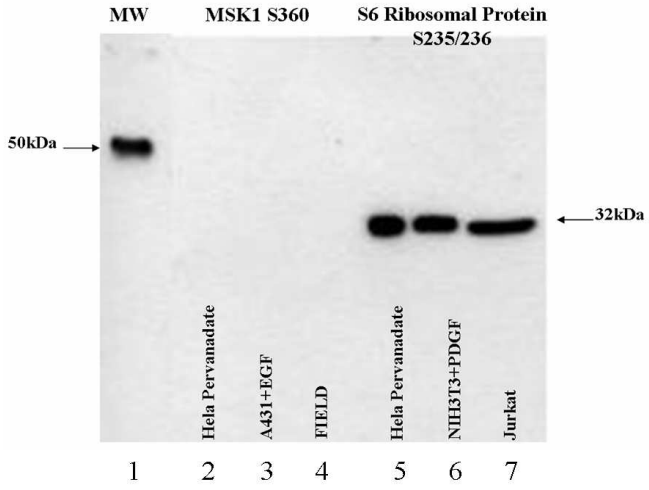

Fig. 2.6: Western Blot example. Unvalidated antibody (with no bands) and validated antibody. Lane 1: MW, molecular weight, visible band at around 50 kDa; lane 2, 3, 4: MSK1 S360, unvalidated antibodies; lane 5, 6, 7: S6 RIBOSOMAL PROTEIN S 235/236, validated antibodies with single band at expected molecular weight of 32kDa.

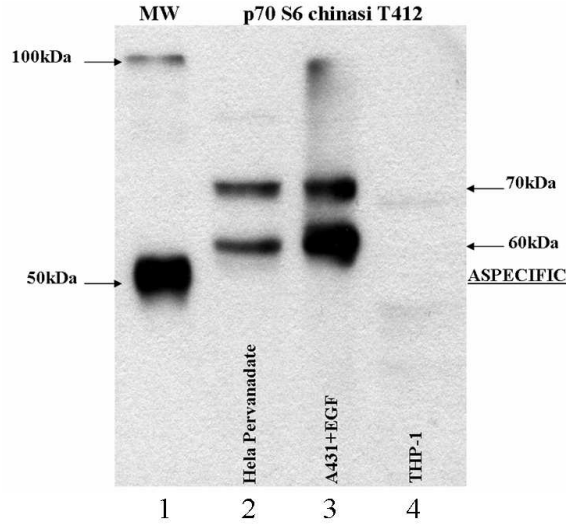

Fig. 2.7: Western Blot example. Unvalidated antibody with aspecific band. Lane 1: MW, molecular weight, two visible bands at around 50kDa and 100kDa, respectively; lane 2, 3, 4: p70 S6 CHINASI T412, unvalidated antibodies with a correct band at expectated molecular weight (70 kDa) and an aspecific band at around 60 kDa.

2.3.4.2 Images Analysis

The TIF images of antibody or Fast Green FCF stained slides were analyzed using the commercially available software, MicroVigene^{TM} Software (VigeneTech Inc, Boston, MA) and protein expression or activation signal was quantified. This software was specifically developed for RPPA analysis and it is able to localize the spots, remove artefacts and subtract the local background, calculating pixel intensity for each spot. The software calculates these values in the antibody stained slides, the corresponding negative control slide (a slide stained only with the secondary antibody) and the Fast Green FCF total protein slide. Then, for each sample, the signal of the negative control array is subtracted from the antibody slide signal and the resulting value is normalized to the total protein value, to ensure that intensity values were not dependent on changes in concentration of printed lysates. Thus, the data analysis generates a normalized single value for every protein studied for each sample.

2.3.5 Western Blot

Total cells lysates were analyzed by SDS-PAGE under reducing conditions. 20μg from total protein fraction were loaded in a precast gel (Criterion, Biorad,

Hercules, CA) and, after gel elettrophoresis run, the proteins were transferred to 0,2μm polyvylidene difluoride membrane (PVDF) (Hybond-P, GE Healthcare, Chalfont St. Giles, UK) following standard methods.

Membranes were saturated for 3 hours with 2% Amersham ECL Advance Blocking Reagent (GE Healthcare) and then incubated overnight at 4°C with primary antibodies. The day after, membranes were washed with PBS1X+Tween 0.1% and then incubated with the secondary antibody for 1 hour at room temperature.

For the signal detection, the immunoreactivity was determinated by an enhanced chemiluminescent reaction (Amersham ECL ADVANCE Western Blotting Detection Kit, GE Healthcare) according to the manufacturer's instructions. For the stripping, membranes were incubated for 15 minutes with a commercial Stripping Solution (Restore Western Blot Stripping Buffer, ThermoScientific, Rockford, IL) and then resaturated for at least 2 hours.

The following antibodies were used for Western Blot at the concentration reported in parenthesis. Primary antibody: anti - PKCalpha S657 (1:1000)(CellSignaling Technology, Inc, Danvers, MA); anti – PKCalpha (1:1000)(Millipore, Billerica, MA), anti - β Actin (1:10.000) (Sigma). Secondary antibodies: HRP-Goat anti-rabbit and anti-mouse IgG-conuigate (1:50000) (Zymed Laboratories; Inc., South San Francisco, CA).

2.3.6 RNA extraction

T-ALL specimens total RNA was isolated using TRIZOL reagent, following manufacturer's instruction (Invitrogen, Paisley, UK). The RNA quality and concentration were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.), respectively.

2.3.7 Real-Time quantitative PCR

2.3.7.1 cDNA synthesis

Reverse transcription–polymerase chain reaction (RT-PCR) amplification was performed. cDNA was synthesized using SuperScriptII reverse transcriptase (Invitrogen) starting from 1μg of total RNA. RNA was primed at 75°C for 10 minutes. Reverse transcriptase reaction was subsequently performed in a total volume of 20μL as following: 25°C for 10 minutes, 42°C for 30 minutes, 99.9°C for 5 minutes.

2.3.7.2 Real-Time quantitative PCR

T-ALL patients *PKCα* expression was quantified with SYBR Green Real-Time Quantitative PCR (RQ-PCR) using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen), by means of 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The housekeeping gene *GUS B* was chosen as reference. Primers were designed with Primer Express Software (Applied Biosystems) to amplify sequences that contain introns, so that spliced mRNA-derived signals could be distinguished from any contaminating genomic DNA-derived signals by their length. To exclude contamination of unspecific PCR products, melting curve analysis was always applied to final PCR products after the cycling protocol. A serial dilution curve of a T-ALL cell line was always amplified in parallel to be used as internal control. cDNA from normal T cells was used as calibrator. *PKCα* expression levels were determinated using the $2^{-\Delta\Delta Ct}$ method.

*PKC*α primers sequences are the following:

- Fwd 5'- GCAAAGGAGCAGAGAACT -3'
- Rev 5'- TACTGCACTCTGTAAGATGG - 3'

*GUS*B primers sequences are the following:

- Fwd 5'-GAAAATATGTGGTTGGAGAGCTCATT-3'
- Rev 5'- CCGAGTGAAGATCCCCTTTTTA-3'.

2.3.8 Microarray Gene expression analysis

All patients included in this study were part of a larger cohort of samples analyzed by Gene Expression Profiling within the "Microarray Innovation in LEukemia" (MILE) study [153]. For microarray experiments *in vitro* transcription, hybridization and biotin labelling were performed according to Roche protocols (Roche Applied Sciences, Indianapolis, IN) developed during the MILE study.

GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) were used to study patients gene expression prolife. Microarray data (CEL files) were generated using default Affymetrix microarry analysis parameters (GCOS 1.2 Software). CEL Files were normalized using robust multiarray averaging expression measure of Affy-R package (http://www.bioconductor.org). CEL Files were then analyzed through Wilcoxon's tests. Multiplicity corrections were used to control false discovery rate; probes with adjusted *p value* lower than 0.05 were declared significant. The multiplicity corrections refer to Benjamini-Hochberg procedure [154].

Ingenuity Pathway Analysis was performed using David Bioinformatics Resources 6.7 in order to identify different genes pathways expression between groups considered.

2.3.9 Statistical analyses

Variance was calculated for each protein analyzed by RPPA, among all expression or activation values of T-ALL patients included in the study. To study the PKCα role in T-ALL, its continuous value was divided into four categories at the 25^{th}, 50^{th}, and 75^{th} percentiles. T-ALL patients were separated into four different groups on the basis of PKCα quartiles and prognosis differences between these groups were analyzed. Relapse free survival was estimated by the Kaplan-Meier analysis and *p values* were calculated by the log rank test. The starting point of the observation time was the date of diagnosis and first relapse was considered as event. In relapse free survival analysis only patients enrolled in LAL 2000-AIEOP treatment protocol were considered.

Wilcoxon tests were performed in order to compare PKCα RPPA values between relapsed and non relapsed patients and to compare *PKCα* mRNA expression values between patients with high and low PKCα RPPA values. Correlation between PKCα RPPA values and mRNA expression values was estimated using Spearman's rank correlation coefficient. To identify clinical or biological features associated with relapsed free survival we fitted an accelerated failure time model (Weibull) with all the variables of interest and backward induction was used to select a best model. To identify clinical and biological factors associated with high or low PKCα RPPA values Chi-square Tests were used. *P values* were corrected to control family wise error rate by means of Holm method. Adjusted *p values* lower than 0.05 were considered statistically significant. A supervised analysis was performed to compare gene expression profile between patients with *PKCα* gene expression lower than the 25^{th} percentile with respect to patients with PKCα gene expression over the 75^{th} percentile and a heatmap was generated to highlight association between the clustering of the patients and that of the genes.

All the analyses were performed using Graphpad Prism version 4 software program (GraphPad Software, La Jolla, CA, USA) or R software package (available at www.r-project.org).

2.3.10 PKCα inhibition in T cell lines

Two selected pediatric T-ALL cell lines, P12 ICHIKAWA and CCRF-CEM, were treated with the commercial PKCα inhibitor Ro-32-0432 (Merk, Darmstadt, Germany), at different concentrations (from 0.5μM to 15 μM). Proliferation changes were studied at various time points by means of MTT assay.

2.3.10.1 MTT assay

The inhibitory activity of Ro-32-0432 on T-ALL cells proliferation was studied using a standard 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazodium bromide (MTT) based assay (Sigma). Cells were seeded at $1x10^4$/well in 96-well microtiter plates and treated with Ro-32-0432 at different concentrations. At several time

points (at 6, 24, 48, 72 hours) cell proliferation was determinated by addiction of 10µL of MTT reagent (5mg/mL). The plate was then incubated at 37°C for 4 hours. Acidic isopropanol (100µl of 0.08N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the formazan crystals. After a few minutes to ensure that all crystals were dissolved, the plate was read on a microtiter plate reader (WALLAC Victor 1420 Spectrophotometer, PerkinElmer Inc., San Jose, CA) using a test wavelength of 570nm and a reference wavelength of 630nm. Absorbance was corrected by subtracting the mean value of non-seeded wells.

2.3.11 *PKCα* gene sequencing

2.3.11.1 PKCα PCR

The DNA of T-ALL patients with high and low PKCα activation in RPPA was amplified by PCR, using specific primers designed on *PKCα* exons of interest by means of Primer Express Software (Applied Biosystems). To study the mutation D294G, the exon coding for S657 phosphorylation site and the exon coding for T497 phosphoryration site, primers sequences are the following:

For D294G:

- Fwd 5'- TGGGACTGGGATCGAAC -3'
- Rev 5'- GAGGAAATTGAAGTCCGTGAG- 3'

For S657:

- Fwd 5'- GCAAAGGAGCAGAGAACT -3'
- Rev 5'- TACTGCACTCTGTAAGATGG - 3'

For T492:

- Fwd 5'- ACTTTGGGATGTGCAAGG -3'
- Rev 5'- CTTGAATGGTGGCTGGATC - 3'.

This reaction was performed in a final volume of 25µL (5µL of DNA + 20µL mix); mix for one sample: 2.5µL Buffer 10X, 0.5µL MgCl2 50mM, 0.5µL dNTPs 10mM, 0.25µL primer forward 20µM, 0.25µL primer reverse 20µM, 0.1µL Taq DNA Polimerase, 17.75µL H_2O).

Following initial denaturation at 94ºC for 3 min, the PCR reaction was carried out for 35 cycles under the following conditions: denaturation at 94ºC for 30 seconds, annealing at 60ºC for 30 minutes, and extension at 72ºC for 30 minutes. The final extension was carried out at 72ºC for 7 minutes.

2.3.11.2 Gene sequencing

PCR products were sequenced by means of automatic sequencer ABI Prism 310 (Applied Biosystems), using the BigDye Terminator Cycle Sequencing method (Applied Biosystems, Foster City, CA, USA). Before sequencing, PCR products were concentrated and purified from PCR reagents through Microcon Centrifugal Filter Devices (Millipore Corporation, BedFord, MA, USA). A purified DNA amount of about 80-90ng was used for a PCR with one of the primers (Forward or Reverse) used for the initial amplification. This reaction was done in a final volume of 20µL (80-90ng of DNA, 4µL of BigDye Mix, 300nM forward or reverse primer). Reaction program: 25 cycles 10 seconds 96°C, 5 seconds 50°C, 2 minutes 60°C. PCR product was then purified by Centri-Sep (Applied BioSysems, Foster City, CA, USA), mixed with 15µL formamide and denaturated at 95°C for 5 minutes. After denaturation, the samples were placed on ice until sequencing.

Sequences output were analyzed by means of FinchTV program (PerkinElmer).

2.4 RESULTS

2.4.1 T-ALL Phosphoproteomic Profiling by RPPA

98 T-ALL pediatric patients at diagnosis were retrospectively analyzed by means of Reverse Phase Protein Arrays technique (RPPA). Patients studied were enrolled in AIEOP treatment protocols. Biological and clinical characteristics of the entire court of T-ALL patients studied by RPPA are resumed in Table A 2.1 in chapter 2.6-Appendix.

Expression or activation of 53 different proteins belonging to different signaling pathways regulating proliferation, apoptosis and cell cycle, was analyzed. Antibodies used in the analyses were previously validated by Western Blot. For reference, antibodies are listed in Table A 2.2 in chapter 2.6-Appendix.

Among all proteins studied by RPPA, PKCα S657 showed a high variance among patients. PKCα S657 RPPA values were continuously distributed among T-ALL samples (Fig. 2.8). PKCα S657 was further studied in depth to investigate its significance in T-ALL.

2.4.2 Cumulative Incidence of Relapse reveals a prognostic significance of PKCα downregulation in T-ALL pediatric patients

T-ALL samples studied by RPPA were divided into four groups on the basis of the quartiles defined on RPPA PKCα S657 values (Fig. 2.9).

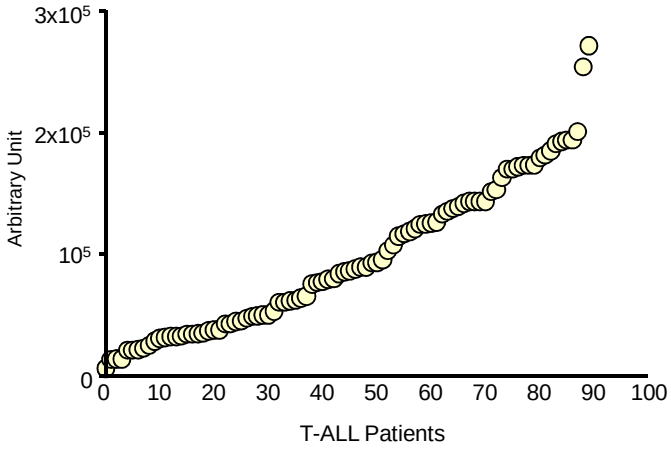

Fig. 2.8: PKCα S657 RPPA values in T-ALL patients studied.

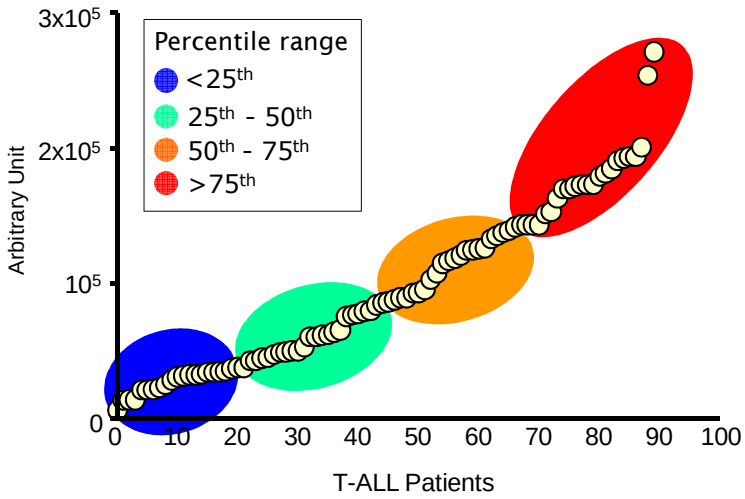

Fig. 2.9: PKCα S657 RPPA values in T-ALL patients divided on the basis of quartiles.

For each quartile a Cumulative Incidence of Relapse analysis was performed through Kaplan Meier estimates and the difference between the curves was assessed by log rank test. Patients with low PKCα S657 RPPA values (under 25th quartile) at diagnosis presented a higher probability to relapse in comparison with patients with high PKCα S657 RPPA values (over 75th quartile). The difference in Cumulative Incidence of Relapse resulted statistically significant (log rank test, p=0.001) (see Fig. 2.10).

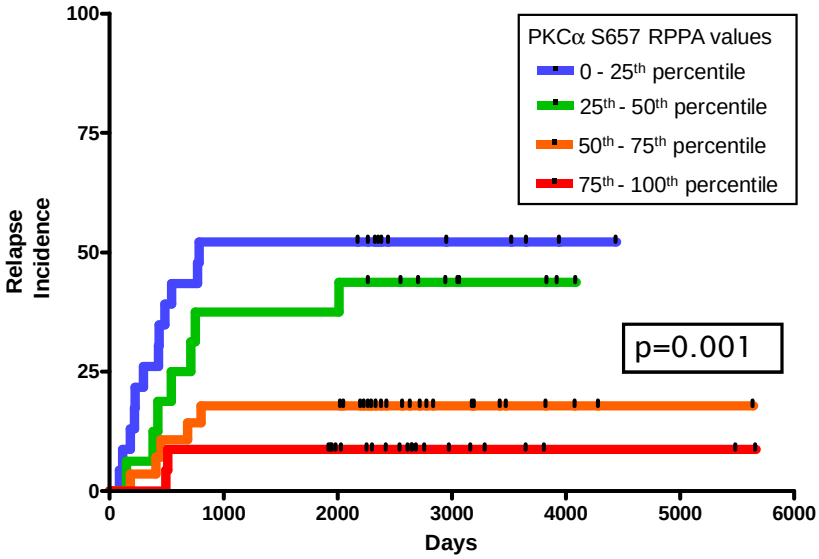

Fig. 2.10: Cumulative Incidence of Relapse on PKCα S657 RPPA values. Cumulative Incidence of Relapse was studied for each group of T-ALL patients divided on the basis of quartiles, defined on PKCα S657 RPPA values. Group characterized by PKCα downregualtion presented a higher probability to replase.

The activation levels of PKCα at diagnosis therefore correlates with T-ALL patients prognosis. In Table 2.1 the percentage of relapsed patients for each quartile is reported.

Table 2.1: T-ALL patients and Relapse incidence. Total number, relapsed and non relapsed patients for each group are reported. The last column reports the percentage of relapse for each quartile of T-ALL patients.

	QUARTILES [Percentage range]	T-ALL patients in each quartile	Relapsed patients	Non relapsed patients	Percentage of relapse
	under 25th	23	12	11	52.2%
	25th – 50th	16	7	9	43.8%
	50th – 75th	28	5	23	17.9%
	over 75th	23	2	21	8.7%

T-ALL patients who did encounter relapse at diagnosis have different values of activated PKCa compared with patients who did not encounter relapse (Wilcoxon Test, p=0.009). This supports the data highlighted by Cumulative Incidence of Relapse analysis (Fig. 2.11).

2.4.3 Multivariate analysis

In order to identify clinical and biological characteristics of T-ALL patients that could be associated to risk of relapse, a multivariate analysis was performed. Patients features considered were sex, phenotype, WBC count, relapse, CNS invasion, Prednisone Response at 8th day of treatment, Minimal Residual Disease.

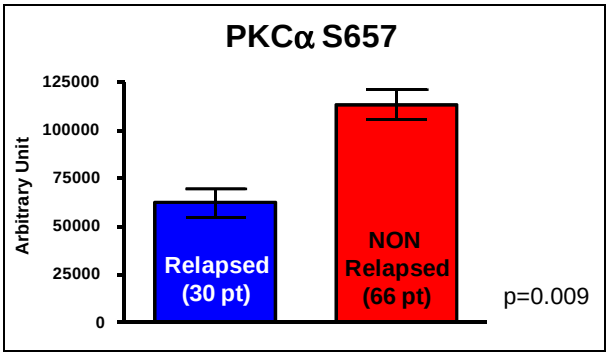

Fig. 2.11: Relapsed vs Non Relapsed T-ALL patients retrospectively studied by RPPA. The comparison between T-ALL patients who encountered relapse versus those who did not encountered relapse revealed a statistically significant difference in PKCα S657. (pt=patients)

This analysis highlights an association between relapse free survival and PKCα S657 levels, categorized over and below the mean (high and low PKCα S657 RPPA values), with an adjusted *p value* of 0.012. Moreover, relapse free survival resulted associated also to Prednisone response (at 8^{th} day of treatment) with an adjusted *p value* of 0.020. A trend of correlation was found between PKCα S657 levels and Prednisone response but not statistically significant (p=0.056). This data suggest that both features, Prednisone response and PKCα S657 values, are associated to relapse free survival, but PKCα seems to be a stronger predictor of risk of relapse.

2.4.4 PKCα S657 levels and clinical/biological patients characteristics

A correlation analysis was performed in order to study the association between PKCα levels (categorized over and below the mean of PKCα S657 RPPA values) and clinical and biological features of T-ALL patients (sex, phenotype, WBC count, relapse, CNS invasion, Prednisone Response at 8^{th} day of treatment, del1p32, Minimal Residual Disease) (See Table 2.2). An association between PKCα S657 levels and relapse incidence was confirmed (Chi-square test; p=0.0389). Relapse incidence resulted to be the only factor that associates to PKCα S657 values with a statistical significance. PKCα could thus be considered a new prognostic biomarker of poor outcome in pediatric T-ALL, never identified before.

Table 2.2: Association between clinical and biological characterists of T-ALL patients and PKCα S657 RPPA values. Statistically significance P<0.05. (Chi square test; Holm multiplicity corrections)

			PKCα RPPA value		
Clinical and molecular features			*low*	*high*	*p value*
Relapse	cohort	90	45	45	
Relapsed					
No.		25	19	6	*0,039*
Rate, %		28%	42%	13%	
Not Relapsed					
No.		65	26	39	*0,039*
Rate, %		72%	58%	87%	
Sex	cohort	90	45	45	
Male					
No.		70	32	38	*1,000*
Rate, %		78%	71%	84%	
Female					
No.		20	13	7	*1,000*
Rate, %		22%	29%	16%	
Phenotype	cohort	90	45	45	
Early T					
No.		35	21	14	*1,000*
Rate, %		39%	47%	31%	
Intermedian/Thymic No.					
No.		44	16	28	*0,321*
Rate, %		49%	36%	62%	
Intermedian/Mature No.					
No.		3	2	1	*1,000*
Rate, %		3%	4%	2%	
Mature No.					
No.		8	6	2	*1,000*
Rate, %		9%	13%	4%	
WBC count	cohort	90	45	45	
< 50000/mm^3					
No.		21	10	21	*1,000*
Rate, %		23%	22%	47%	
> 50000/mm^3					
No.		59	35	24	*1,000*
Rate, %		66%	78%	53%	
CNS invasion	cohort	90	45	45	
YES					
No.		10	5	5	*0,961*
Rate, %		11%	11%	11%	
NO					
No.		80	40	40	*0,961*
Rate, %		89%	89%	89%	
PDN response (8th day of treatment)	cohort	90	45	45	
Poor (leukemic blasts>1000/µL)					
No.		31	20	11	*1,000*
Rate, %		34%	44%	24%	
Good (leukemic blasts<1000/µL)					
No.		59	25	34	*1,000*
Rate, %		66%	56%	76%	
del1p32	cohort	82	43	39	
YES					
No.		5	6	1	*1,000*
Rate, %		6%	14%	3%	
NO					
No.		78	37	38	*1,000*
Rate, %		95%	86%	97%	
Minimal Residual Disease	cohort	83	42	41	
Standard Risk					
No.		15	6	9	*0,233*
Rate, %		18%	14%	22%	
Medium Risk					
No.		48	23	25	*0,494*
Rate, %		58%	55%	61%	
High Risk					
No.		20	13	7	*1,000*
Rate, %		24%	31%	17%	

2.4.5 Validation of RPPA data by Western Blot

RPPA data were validated by Western Blot in an independent group of patient samples at diagnosis of T-ALL who did or did not encounter relapse. PKCα S657 resulted downregulated in the relapsed patients group, confirming the RPPA results (Fig. 2.12).

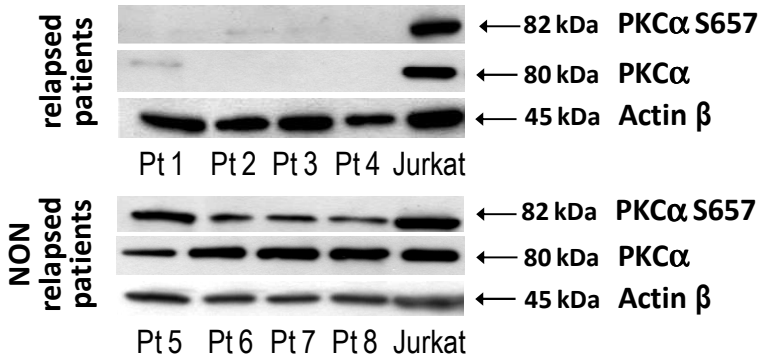

Fig. 2.12: RPPA data validation. PKCα S657 downregulation in relapsed T-ALL patients was confirmed by Western Blot (82kDa). Also total PKCα (80kDa) and Actinβ (45kDa) were studied.

PKCα total protein level also resulted different between the two groups. PKCα protein expression was lower in patients who relapsed, in concordance with the activation levels of the kinase. Thus, the hypoactivation of PKCα S657 observed in relapsed patients in RPPA seems to derive from a low PKCα total protein expression, that might be due to a reduced *PKCα* gene expression.

2.4.6 *PKCα* mRNA expression in T-ALL

In order to investigate the *PKCα* mRNA expression levels in T-ALL, a RQ-PCR analysis was first performed in 12 patients with low PKCα S657 and 12 patients with high PKCα S657 in RPPA.

RQ-PCR analyses revealed downregulation of *PKCα* mRNA in T-ALL patients who presented low PKCα activation in RPPA (Wilcoxon test, $p<0.0001$) (see Fig. 2.13).

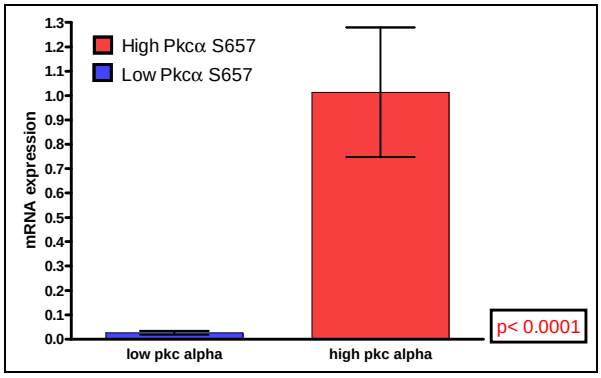

Fig. 2.13: *PKCα* mRNA expression in T-ALL patients. 12 patients with low PKCα S657 and 12 patients with high PKCα S657 in RPPA were studied by RQ-PCR for *PKCα* mRNA expression. It resulted higher in patients with high PKCα S657 RPPA values (Wilcoxon test, p<0.0001).

PKCα mRNA expression in T-ALL patients was compared with *PKCα* mRNA expression of healthy T cells. In patients with low PKCα S657 levels in RPPA, *PKCα* mRNA expression resulted downregulated with respect to healthy T cells. On the other hand, patients with high PKCα activation in RPPA showed *PKCα* mRNA levels similar to healthy control (Fig. 2.14).

Thus, a different *PKCα* mRNA expression was confirmed between these two groups of patients, supporting the thesis that the difference of PKCα S657 levels lies in a different *PKCα* gene expression. The different activation and expression of PKCα protein initially observed is thus a consequence of a dissimilar expression of the *PKCα* gene between the two T-ALL groups.

A Spearman analysis was performed in order to correlate *PKCα* mRNA

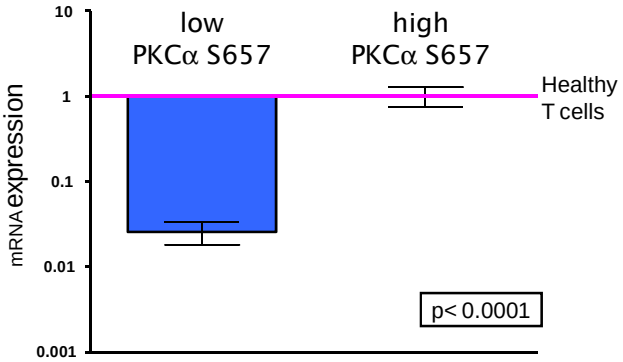

Fig. 2.14: *PKCα* mRNA expression in T-ALL patients compared to healthy T cells. Patients with low PKCα levels in RPPA showed a *PKCα* mRNA downregulation with respect to its expression in control T cells. Patients with high PKCα activation in RPPA presented *PKCα* mRNA levels similar to the healthy control.

expression to PKCα S567 RPPA data and revealed a statistically significant correlation (Spearman ρ=0.81; p=1.448e-06) (Fig. 2.15). 24 T-ALL patients, previously studied by RPPA and then analyzed by RQ-PCR, were considered for Spearman analysis.

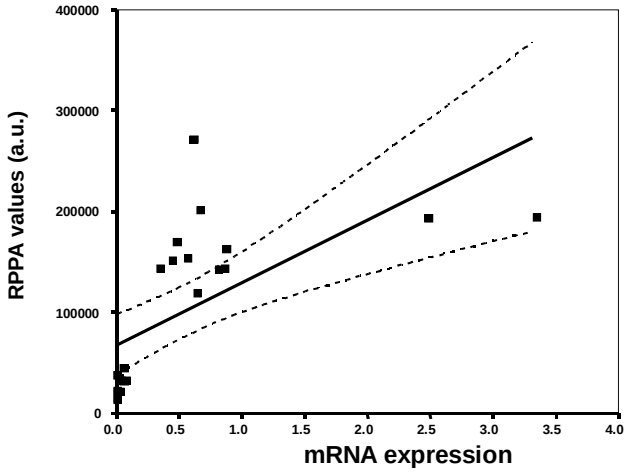

Fig. 2.15: Correlation between PKCα protein expression and mRNA expression. Low protein activation levels resulted correlated to low mRNA expression values. Spearman correlation: ρ=0.81 (p=1.448e-06).

2.4.7 Gene Expression Profiling of T-ALL pediatric patients

To deepen the investigation on *PKCα* gene expression in T-ALL, a gene expression profiling study of 90 patients was performed by the means of HG-U133 Plus 2.0 arrays on Affymetrix platform. All patients included in this study were part of a larger cohort of samples analyzed by Gene Expression Profiling within the "Microarray Innovation in LEukemia" (MILE) study [153].

Analysis of *PKCα* gene expression revealed continous values among all patients. To identify genes correlated with different *PKCα* expression, the T-ALL cohort was divided into four groups defined on the basis of quartiles on *PKCα* expression values. A supervised analysis was performed between patients with *PKCα* gene expression lower than the 25th percentile and those with PKCα gene expression over the 75th percentile (Wilcoxon test). Two different signatures were identified among these patients (See Fig. 2.16). Between two groups of patients, 567 probe sets were differentially expressed. In Table A 2.3, chapter 2.6-Appendix, probes with a fold change higher than 1.5 are listed.

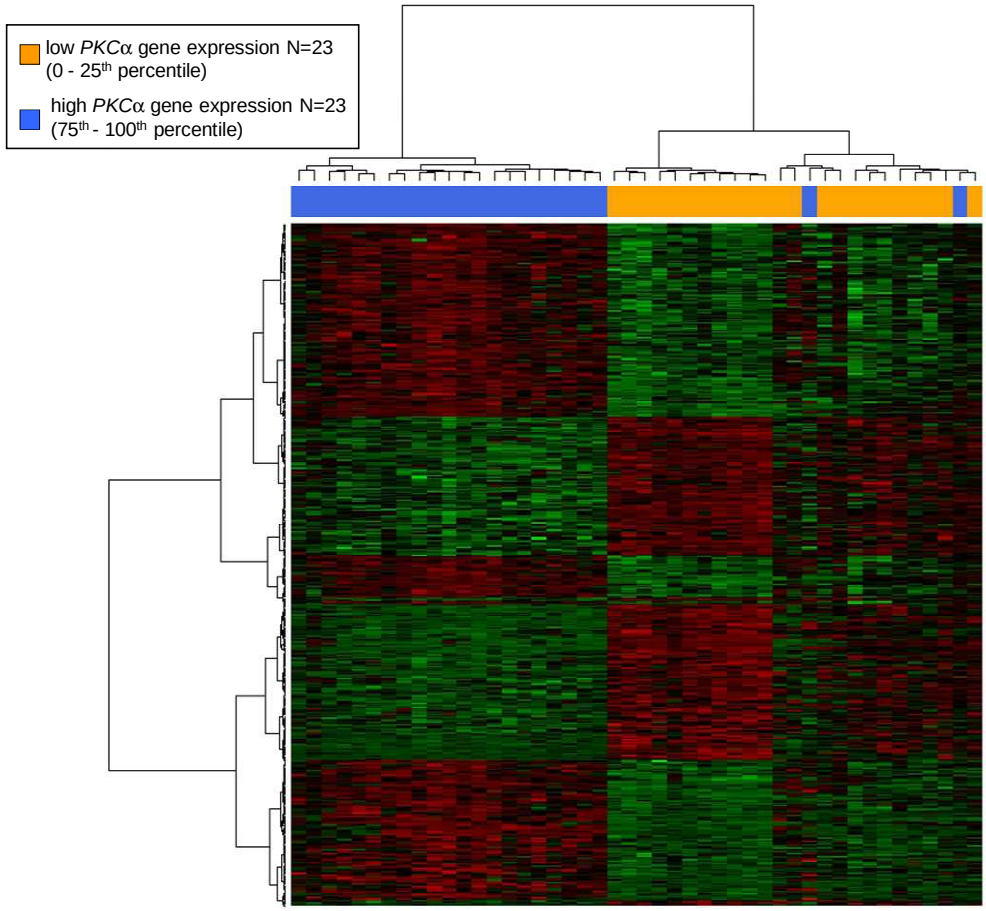

Fig. 2.16: Supervised Analysis of 90 T-ALL patients. Gene expression profiling was performed using HG-U133 Plus 2.0 arrays on Affymetrix platform. Patients with low *PKCα* gene expression presented a different signature compared to those with high *PKCα* gene expression.

Patients with high *PKCα* expression showed an upregulation of genes belonging to the phosphatidylinositol signalling pathway through gene pathway analysis (see Table 2.3). This data is very important because these genes are involved in the production of DAG and Ca^{2+}, the main PKCα cofactors that play an essential role in mediating its translocation from cytosol to the plasmatic membrane and its consequent activation.

In order to investigate if *PKCα* gene expression is also associated to prognosis in T-ALL, as already found with RPPA PKCα activation data, a Cumulative Incidence of Relapsed was performed.

Table 2.3: Genes belonging to phosphatidylinositol signaling pathway resulted differentially expressed in groups with high and low PKCα gene expression. Patients with high PKCα expression showed an upregulation of genes belonging to phosphatidylinositol signalling pathway through gene pathway analysis.

AFFYMETRIX_3PRIME_IVT_ID	GENE NAME	Related Genes	Species
202660_at, 202662_s_at, 202661_at	inositol 1,4,5-triphosphate receptor, type 2	RG	Homo sapiens
228006_at	phosphatase and tensin homolog; phosphatase and tensin homolog pseudogene 1	RG	Homo sapiens
242494_at, 213222_at, 244726_at	phospholipase C, beta 1 (phosphoinositide-specific)	RG	Homo sapiens
203895_at, 203896_s_at	phospholipase C, beta 4	RG	Homo sapiens
213093_at, 206923_at, 215195_at	protein kinase C, alpha	RG	Homo sapiens

This analysis revealed a different percentage of patients who encounted relapse in the group with low *PKCα* gene expression (lower the 25th quartile, N=23; 43.5% relapsed) compared to that of patients with high *PKCα* gene expression (over the 75th quartile, N=23; 13% relapsed): patients with low *PKCα* gene expression have a higher probability to relapse. The difference in Cumulative Incidence of Relapse resulted statistically significant (log rank test, p=0.02) (Fig. 2.17). This result corroborates the finding that PKCα expression is prognostic significant in T-ALL reported by RPPA data as well, underlining the novel role of this kinase in prediction of T-ALL prognosis.

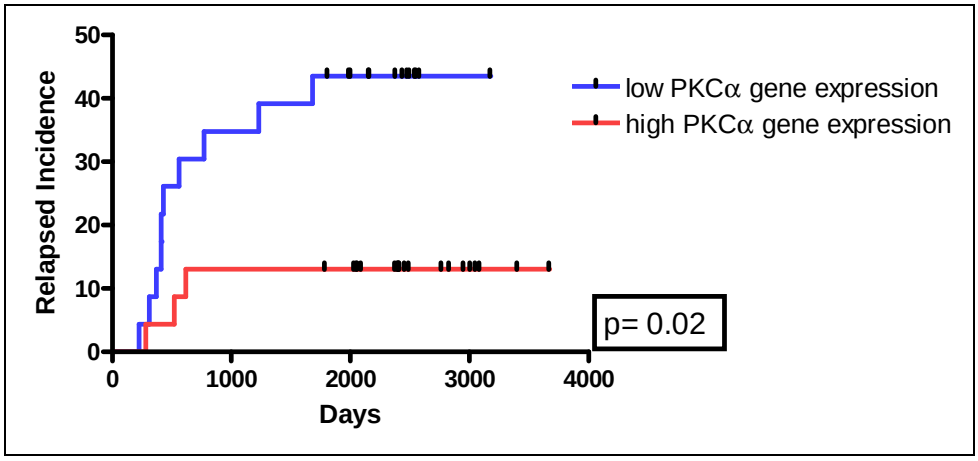

Fig. 2.17: Cumulative Incidence of Relapse *PKCα* gene expression values. Patients with low *PKCα* gene expression showed a higher probability to encounter relapse compared to those with high *PKCα* gene expression.

2.4.8 PKCα inhibition in T leukemic cell lines induces an increase in proliferation rate

In order to analyse the functional role of PKCa in regulating cell proliferation, we at first analyzed PKCα and PKCα S657 expression by Western Blot in 5 T leukemic cell lines (DND41, P12 ICHIKAWA, MOLT3, TALL1 and CCRF-CEM) (see Fig. 2.18). Two pediatric leukemia cell lines, P12 ICHIKAWA and CCRF-CEM were selected as *in vitro* model for PKCα inhibition experiments.

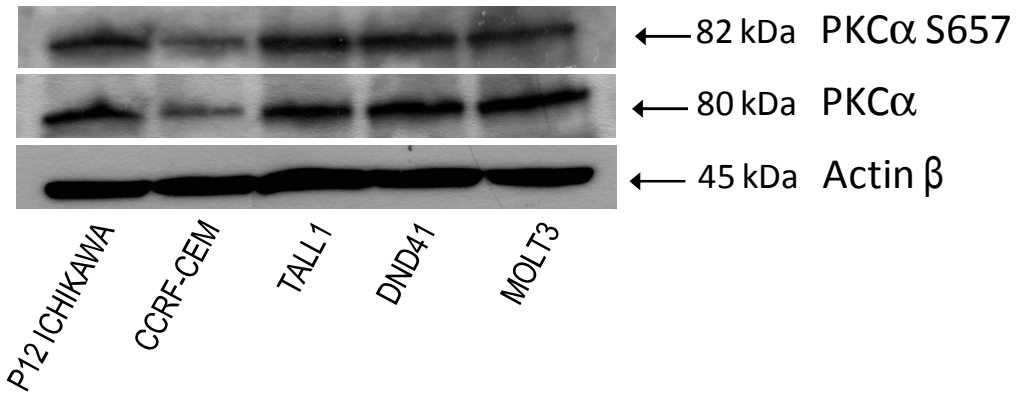

Fig. 2.18: Western Blot analysis in T leukemic cell lines. PKCα S657 (82kDa), total PKCα (80kDa) and Actin β (45kDa) were studied in five T leukemic cell lines.

These cell lines were treated with a commercial PKCα inhibitor, Ro-32-0432, at different concentrations (from 0.5µM to 15µM) and proliferation was studied by the means of the MTT assay at several time points (6, 24, 48, 72 hours). An increase in cell proliferation was observed in each cell line at the lowest concentrations (0.5 and 1µM) after 48h of the treatment (Wilcoxon test, $p<0.05$ for treatment at 0.5µM) (Fig. 2.19). Inhibition of PKCα induces an increase in T leukemic cell lines proliferation, according to the thesis which supports that PKCα downregulation leads to a more aggressive T leukemic cells phenotype, reflecting the condition of patients who experience relapse.

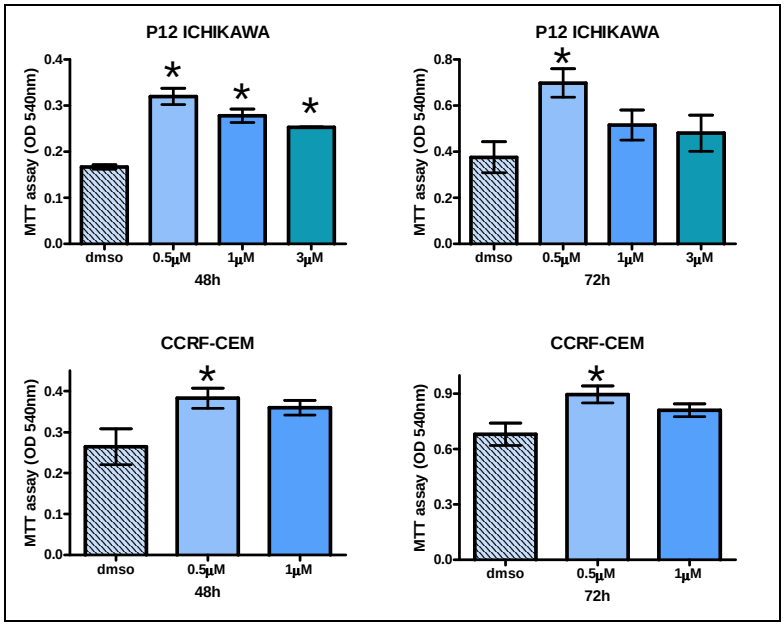

Fig. 2.19: PKCα inhibition in T leukemic cell lines: increase of proliferation. T leukemic cell lines P12 ICHIKAWA and CCRF-CEM were treated with commeracial PKCα inhibitor RO-32-0432 at different concentrations (from 0.5μM to 15μM) and proliferation rate was studied by MTT Assay. In figure proliferation data of treatment at 0.5μM and 1μM are reported. (* : $p<0.05$).

2.4.9 Sequencing of PKCα gene

A set of T-ALL patients, already studied by RPPA, who presented low PKCα activation and a group with high PKCα activation was screened by DNA sequencing in order to search for the presence of mutations in the *PKCα* gene.

Zhu Y. et al [155] described a single point mutation (D294G) in *PKCα* in pituitary and thyroid tumors associated to a more invasive phenotype. This loss of function mutation abolished tightly binding of PKCα D294G mutants to the cellular membrane. This impaired membrane binding has been associated to the mutants inability to transduce several antitumorigenic signals.

The sequencing analysis focused in particular on the study of the mutation at D294G and of *PKCα* exons coding for the phosphorylation sites. No mutations were detected in the *PKCα* gene for both groups of T-ALL patients.

2.5 DISCUSSION

T-lineage Acute Lymphoblastic Leukemia (T-ALL) prognosis in pediatric patients has improved in the last few decades. Intensified treatments achieved five-years-relapse-free survival rates of about 75%, but the every forth patient still encounters relapse, in particular early relapse [32]. It is therefore extremely important to gain insight in the molecular basis of therapy resistance in these patients, in order to identify new candidate prognostic markers and aberrant therapeutically targetable molecules. The deeper understanding of T-ALL molecular players and leukemogenic pathways will allow the development of novel more specific and less toxic therapies and the identification of new prognostic factors to improve T-ALL patients stratification on the basis of more reliable parameters. Indeed, even if many genetic aberrations have been correlated to T-ALL prognosis in retrospective studies, no clear association is defined between candidate prognostic factors and patients outcome. In this study, proteins activation and expression in pediatric T-ALL at diagnosis have been investigated by means of the high throughput technique Reverse Phase Protein Arrays (RPPA). Protein network activation state in 98 T-ALL pediatric patients samples at diagnosis was retrospectively analyzed using RPPA. Expression or activation of fifty-three different proteins/phosphoproteins has been analyzed. From this screening, PKCα S657 has been identified as an interesting protein to be studied in depth: its activation in analyzed patients samples presented continuous values with a high variance among T-ALL patients.

PKCα is a protein kinase expressed in many tissues and associated with a number of biological functions such as cell proliferation, differentiation, cell cycle control, apoptosis, cell survival, cell adhesion and cell motility. PKCα activation induces different cellular responses depending on its temporal activation, downstream targeted pathways and tissue specifity. In cancer, PKCα has been described as tumor suppressor as well as having an oncogenic role [84][85]. Specimens studied by RPPA were thus divided into four groups on the basis of PKCα S657 values quartiles. A relapse free survival analysis revealed that

patients who presented a downregulation of PKCα S657 in RPPA have a higher incidence of relapse. In particular, 52.2% of patients under 25th quartile experienced relapse versus 8.7% of patients over 75th quartile. This data suggests a potential prognostic role of PKCα S657 levels in T-ALL never described before.

In order to identify clinical and biological characteristics of T-ALL patients that could be related to relapse occurrence, a multivariate analysis was performed. PKCα S657 RPPA values, categorized on the basis of the mean, were considered among the clinical and biological patients features (sex, phenotype, WBC count, relapse, CNS invasion, Prednisone Response at 8th day of treatment, Minimal Residual Disease) and related to relapse free survival data. Results revealed that PKCα S657 RPPA values is the most relevant factor related to prognosis with the highest statistically significance (p=0.012).Also Prednisone response at 8th day of treatment, one of the strongest prognostic factor in childhood ALL [156], showed a good association with relapse free survival (p=0.020), even if less significant that PKCα S657. Thus, we interrogated the relation between PKCα S657 levels and Prednisone response and a correlation analysis was performed. A trend of correlation was found between PKCα S657 levels and Prednisone response (p=0.056). These data suggest that both features, Prednisone response and PKCα S657 values, are associated with relapse free survival, but PKCα RPPA values resulted to be the strongest predicting factor of relapse at diagnosis in T-ALL pediatric patients. Interestingly, among the described prognostic markers already considered in clinical practice, also WBC count [156] is a very important one, but we did not find an association with relapse free survival in our patients cohort. No association was also found between relapse free survival and T-ALL different maturation stages, CNS infiltration and Miminal Residual Disease (MRD) final stratification. This latter data seems not to confirm data presented by Schrappe M *et al.* (2011) [157]. Unfortunately, no data were available to study the correlation of relapse free survival and residual disease on day 15 in the bone marrow, a strong predictor of relapse risk in childhood ALL [158]. A relevant aspect to examine would also be the association between PKCα and NOTCH1

mutations, since NOTCH1 activating mutations have been described in 50-60% of T-ALL and seem to have a disputed prognostic role [159]. We are currently collecting data of NOTCH1 mutations in our cohort of pediatric T-ALL patients.

PKCα S657 relevance in predicting T-ALL relapse was further confirmed comparing RRPA data of patients that encountered relapse with those of specimens who did not encountered relapse in a Wilcoxon test. Among all proteins studies, PKCα S657 was differentially activated between these two groups of patients. RPPA results were validated by Western Blot in an independent set of T-ALL patients. PKCα can thus be considered as a new prognostic biomarker of poor outcome in pediatric T-ALL.

To investigate if different expression of PKCα S657 could depend on different levels of total PKCα, we analyzed the expression of total PKCα protein by Western Blot. A difference between relapsed and non relapsed patients in PKCα protein expression was detected. PKCα total protein was lower in patients who relapsed, in concordance with the activation levels of the kinase. Thus, the hypoactivation of PKCα S657 observed in relapsed patients in RPPA seems to derive from a low PKCα total protein expression, that could be caused by a reduced *PKCα* gene expression.

In order to understand if the difference in PKCα protein levels could be a consequence of a gene expression downregulation, a Real time Quantitative PCR (RQ-PCR) analysis was performed in 24 T-ALL patients (12 patients with high and 12 patients with low PKCα S657 values), previously studied by RPPA. A downregualtion in *PKCα* mRNA expression was detected in patients with low PKCα activation in RPPA. Moreover, mRNA expression data resulted to be correlated with RPPA data through a Spearman correlation analysis (Spearman $\rho=0.81$; $p=1.448e-06$).

To deepen the investigation on *PKCα* gene expression in T-ALL, we analyzed *PKCα* probe set expression in a cohort of 90 patients previously studied by Gene Expression Profiling within the “Microarray Innovation in LEukemia” (MILE) study [153]. Analysis of *PKCα* gene expression revealed continuous values among 90 T-ALL patients. The cohort was then divided into four groups defined

on the basis of *PKCα* expression quartiles and a supervised analysis was performed between patients with *PKCα* gene expression lower than the 25th percentile and those with *PKCα* gene expression over the 75th percentile. Between these two groups of patients, 567 probe sets resulted to be differentially expressed. In particular, an upregulation of genes belonging to phosphatidylinositol signalling pathway was identified in patients with high *PKCα* expression through gene pathway analysis This data is very important because these genes are involved in the production of DAG and Ca^{2+}, the main PKCα cofactors that play an essential role in mediating its translocation from cytosol to the plasmatic membrane and its consequent activation. Further studies will clarify the involvement of these genes in PKCα activation in T-ALL, deepening the functional aspects of PKCα regulation in T leukemic cells.

In order to investigate if also *PKCα* gene expression associated to prognosis in T-ALL, as already found with RPPA PKCα activation data, a Cumulative Incidence of Relapse was performed. A higher percentage of patients who encountered relapse was found in the group with low *PKCα* gene expression (lower the 25th quartile, N=23; 43.5%) in comparison to patients with high *PKCα* gene expression (over the 75^{th} quartile, N=23; 13%). This result showed that patients with low *PKCα* gene expression have a higher probability to relapse. Through these analyses we confirmed the prognostic significance of PKCα expression in T-ALL, as revealed from RPPA data. PKCα can be thus proposed as a novel marker for relapse prediction at diagnosis of T-ALL.

These new results in childhood T-lineage acute leukemia needs to be validated in a larger clinical set. In order to confirm the relevance of *PKCα* expression levels at diagnosis for relapse prediction, a wide screening of *PKCα* mRNA expression by RQ-PCR analysis has been planned. This analysis will be performed in a large cohort (at least 200 patients) of T-ALL pediatric patients at diagnosis enrolled in the LAL 2000 - AIEOP treatment protocol. Once confirmed, we will be able to propose *PKCα* as a new prognostic factor for clinical diagnosis of T-ALL.

Moreover, we also intend to investigate *PKCα* expression in a set of Early T-cell Precursor Acute Lymphoblastic Leukemia (ETP-ALL) patients. ETP-ALL comprises up to 15% of T-ALL and it is associated to a high risk of treatment failure. ETP-ALL is characterized by a specific very immature immunophenotype (lack of expression of the T-lineage surface markers CD1a and CD8, weak or absent expression of CD5, aberrant expression of myeloid and hematopoietic stem cell markers, such as CD13, CD33, CD34 and CD117). It would be important to understand if ETP-ALL patients are also characterized by low *PKCα* expression levels at diagnosis [17].

Another important aspect to be investigated concerns causes and effects of PKCα downregulation. Such studies could help identify new therapeutic targets for a more specific and effective treatment. A functional study of the PKCα role in T leukemic cells has been taken up with the *in vitro* inhibition of PKCα activity and the evaluation of the effects on proliferation rate. Two pediatric cell lines were selected as *in vitro* models and treated with a PKCα commercial inhibitor, Ro-32-0432, at different concentrations. Proliferation was then studied at several time points by means of the MTT assay. An increase in cell proliferation was observed in treated cell lines at the lowest concentrations (0.5 and 1μM) after 48h of treatment. Inhibition of PKCα thus showed to induce an increase in proliferation of T leukemic cell lines and this is in agreement with our findings of downregulation of *PKCα* in patients with a more aggressive disease. Together with specific *in vitro* inhibition, studies in progress concern *PKCα* gene silencing through lentiviral transfection of two specific short hairpin RNAs (shRNAs). P12 ICHIKAWA and CCRF-CEM cells lines will be transduced and proliferation rates and cell cycle progression will be analyzed. More proteomics studies will be also performed on silenced cells in order to identify targets that are regulated downstream of PKCα. This will enable us to elucidate the signalling pathways affected by PKCα downregulation and thus to identify new potential therapeutic targets.

Finally, it will be of interest to also investigate the origin of low *PKCα* gene expression in T-ALL patients i.e. by a gene promoter methylation studies or a micro-RNA expression profile analysis.

In this study, a new promising prognostic biomarker in childhood T-ALL had been discovered through a retroprospective analysis of phosphoproteomic profiles by means of RPPA. PKCα resulted to be downregulated at diagnosis in T-ALL patients who experienced relapse. Lower levels of both PKCα protein activation and expression were detected in T-ALL patients with a higher incidence of relapse. Multivariate analysis revealed that, among clinical and biological patients characteristics, only PKCα levels and Prednisone response at 8^{th} day of treatment were related to relapse free survival. In particular, in this study PKCα showed a stronger relation to relapse than Prednisone response. This information leads us to propose PKCα as a new prognostic factor in pediatric T-ALL, but screening of PKCα expression in a larger clinical set of T-ALL patients is needed.

Moreover, PKCα protein downregulation was shown to derive from a lower mRNA expression of *PKCα* gene by RQ-PCR analysis. This data was confirmed in a larger cohort of T-ALL pediatric patients studied by Gene Expression Profiling. Functional studies on PKCα inhibition effects in T leukemic cell lines are on going and preliminary results showed an increase in proliferation rate after treatment, in agreement with our findings of a downregulation of *PKCα* in patients with an aggressive disease. To confirm this data, studies in progress of *PKCα* gene silencing through lentiviral transfection of two specific shRNA will let us to analyze proliferation rates and cell cycle progression and to identify new aberrant targets in T-ALL cells.

2.6 APPENDIX

Table A 2.1: Clinical and biological features of T-ALL patient studied by RPPA.

Characteristic	*Number*	*%*
Sex		
male	75	76,5
female	23	23,5
Age (years)		
<5	18	18,4
5-10	44	44,9
>10	36	36,7
AIEOP[a] therapy protocol		
ALL[b] 88	1	1
ALL 91	5	5,1
ALL 95	10	10,2
ALL 2000	82	83,7
Immunophenotype T-ALL		
early	35	35,7
thymic	42	42,9
intermediate	5	5,1
intermediate/mature	4	4,1
mature	12	12,2
WBC count		
>50000/mm^3	64	66,7
<50000/mm^3	32	33,3
ND	2	
DNA Index		
1-1.16	82	97,6
>1.16	2	2,4
ND[c]	14	
Prednisone Response (8th day of treatment)		
good (leukemic blasts<1000/µL)	59	63,4
poor (leukemic blasts>1000/µL)	34	36,6
ND	5	
CNS invasion		
yes	10	10,3
no	87	89,7
ND	1	
MRD (only AIEOP LLA 2000 patients)		
standard risk	15	18,1
intermedium risk	48	57,8
high risk	20	24,1
ND	15	
Outcome		
dead	29	29,6
CR[d]	69	70,4
Relapse		
yes	30	31,3
no	66	68,7
ND	2	
del1p32		
yes	7	8,5
no	75	91,5
ND	16	
t(10;11)		
yes	7	12,1
no	51	87,9
ND	40	

[a]AIEOP= Associazione Italiana Ematologia Oncologia Pediatrica.
[b]ALL= Acute Lymphoblastic Leukemia.
[c]ND= No Data.
[d]CR= Complete Remission

Table A 2.2: Antibodies used in T-ALL RPPA study. Catalog number, molecolar weight, company, host, dilution used in Westen Blot, dilution used in RPPA staining are reported.

Antibody	Catalog number #	MW (kDa)	Company	Host	Dilution (WB)	Dilution (RPPA)
4E-BP1 (S65)	9451	15-20	CellSignaling	Rabbit	1:1000	1:100
Akt (S473)	9271	60	CellSignaling	Rabbit	1:1000	1:100
Akt (T308)	9275	60	CellSignaling	Rabbit	1:1000	1:100
Annexin II	610068	36	BD	Mouse	1:2000	1:500
A-Raf (S299)	4431	68	CellSignaling	Rabbit	1:500	1:50
Bak	06-536	30	Upstate	Rabbit	1:1000	1:1000
Bax	2772	20	CellSignaling	Rabbit	1:1000	1:250
Bcl-2 (S70)	2827	28	CellSignaling	Rabbit	1:1000	1:250
Bcl-2 (T56)	2875	28	CellSignaling	Rabbit	1:1000	1:200
Bcl-xL	2762	30	CellSignaling	Rabbit	1:1000	1:500
B-Raf (S445)	2696	95	CellSignaling	Rabbit	1:500	1:50
Caspase-6, cleaved (D162)	9671	18	CellSignaling	Rabbit	1:1000	1:50
Caspase-7, cleaved (D198)	9491	20	CellSignaling	Rabbit	1:1000	1:1000
Caspase-9, cleaved (D330)	9501	17, 37	CellSignaling	Rabbit	1:1000	1:50
CDK2 (78B2)	2546	33	CellSignaling	Rabbit	1:1000	1:100
Cleaved NOTCH1 Val1744	2421	110	CellSignaling	Rabbit	1:1000	1:100
CREB (S133)	9191	43	CellSignaling	Rabbit	1:1000	1:100
Cyclin A clone BF683	05-374	58	Upstate	Mouse	1:1000	1:100
Cyclin B	610220	62	BD	Mouse	1:1000	1:100
Cyclin D1 (G124-326)	554180	36	BD	Mouse	1:1000	1:20
Cyclin E (HE12)	554182	50	BD	Mouse	1:1000	1:100
eNOS/NOS III (S116)	07-357	132	Upstate	Rabbit	1:1000	1:500
ERK 1/2 (T202/Y204)	9101	42, 44	CellSignaling	Rabbit	1:1000	1:2000
FADD (S194)	2781	28	CellSignaling	Rabbit	1:1000	1:250
FAK (Y397) (18)	611806	125	BD	Mouse	1:1000	1:50
GRB2	3972	25	CellSignaling	Rabbit	1:1000	1:1000
GSK-3alpha/beta (S21/9)	9331	46, 51	CellSignaling	Rabbit	1:1000	1:100
HSP70 (C92F3A-5)	SPA-810	70	Stressgen	Mouse	1:2000	1:200
IRS-1 (S612)	2386	180	CellSignaling	Rabbit	1:1000	1:50
JAK1 (Y1022/1023)	3331	130	CellSignaling	Rabbit	1:1000	1:200
KIP1/p27 (57)	610241	27	BD	Mouse	1:5000	1:100
LCK (Y505)	2751	56	CellSignaling	Rabbit	1:1000	1:500
MEK1/2 (S217/221)	9121	45	CellSignaling	Rabbit	1:1000	1:400
mTOR (S2448)	2971	289	CellSignaling	Rabbit	1:1000	1:100
NF-kappaB p65 (S536)	3031	65	CellSignaling	Rabbit	1:1000	1:50
p38 MAP KINASE (T180/Y182)	9211	40	CellSignaling	Rabbit	1:1000	1:100
p70 S6 KINASE (T389)	9205	70, 85	CellSignaling	Rabbit	1:1000	1:20
p90RSK (S380)	9341	90	CellSignaling	Rabbit	1:1000	1:250
PARP, cleaved (D214)	9541	89	CellSignaling	Rabbit	1:1000	1:100
PDK1 (S241)	3061	63	CellSignaling	Rabbit	1:1000	1:200
PKA C (T197)	4781	42	CellSignaling	Rabbit	1:1000	1:100
PKC alpha (S657)	06-822	82	Upstate	Rabbit	1:500	1:1500
PKC alpha/beta II (T638/641)	9375	80, 82	CellSignaling	Rabbit	1:1000	1:100
PKC delta (T505)	9374	78	CellSignaling	Rabbit	1:1000	1:100
PKC theta (T538)	9377	79	CellSignaling	Rabbit	1:1000	1:100
PTEN (S380)	9551	54	CellSignaling	Rabbit	1:1000	1:500
S6 RIBOSOMAL PROTEIN (S235/236) (2F9)	4856	32	CellSignaling	Rabbit	1:1000	1:200
SAPK/JNK (T183/Y185)	9251	46, 54	CellSignaling	Rabbit	1:1000	1:200
SMAC/DIABLO	2954	21	CellSignaling	Mouse	1:1000	1:5000
STAT1 (Y701)	9171	84, 91	CellSignaling	Rabbit	1:1000	1:50
STAT3 (S727)	9134	79, 86	CellSignaling	Rabbit	1:1000	1:200
STAT5 (Y694)	9351	90	CellSignaling	Rabbit	1:1000	1:50
ZAP-70 (Y319)/ SYK (Y352)	2717	70, 72	CellSignaling	Rabbit	1:1000	1:100

Table A 2.3: Up- and Down- regulated probe sets and genes identified in T-ALL specimens under 25th quartile of PKCalpha gene expression vs T-ALL specimens over 75th quartile of PKC alpha gene expression (only probes set with a fold change higher that 1.5 are reported).

probe set	gene symbol	lfdr (local false discovery rate)	group under 25th quartile means	group over 75th quartile means	fold change
213093_at	PRKCA	$43{,}3 \times 10^{-9}$	5,21	10,72	0,49
225782_at	MSRB3	$478{,}4 \times 10^{-6}$	4,61	8,68	0,53
224802_at	NDFIP2	$12{,}1 \times 10^{-3}$	4,61	7,87	0,59
1556945_a_at	NA	$910{,}7 \times 10^{-6}$	5,04	8,55	0,59
229975_at	BMPR1B	$1{,}5 \times 10^{-3}$	4,73	7,88	0,60
203895_at	PLCB4	$478{,}4 \times 10^{-6}$	4,62	7,55	0,61
227530_at	AKAP12	$36{,}9 \times 10^{-3}$	4,70	7,67	0,61
1556944_at	NA	$4{,}5 \times 10^{-3}$	4,69	7,57	0,62
242579_at	BMPR1B	$2{,}9 \times 10^{-3}$	4,69	7,56	0,62
227001_at	NIPAL2	$478{,}4 \times 10^{-6}$	4,36	6,75	0,65
225790_at	MSRB3	$4{,}6 \times 10^{-3}$	4,47	6,86	0,65
215784_at	CD1E	$8{,}7 \times 10^{-3}$	7,61	11,67	0,65
1566482_at	NA	$17{,}4 \times 10^{-3}$	4,02	6,11	0,66
215195_at	PRKCA	$43{,}3 \times 10^{-9}$	5,19	7,87	0,66
224799_at	NDFIP2	$37{,}5 \times 10^{-3}$	4,67	7,06	0,66
1561195_at	NA	$24{,}3 \times 10^{-6}$	7,91	5,15	1,54
1561985_at	C14orf39	$17{,}4 \times 10^{-3}$	6,10	3,96	1,54
206159_at	GDF10	$131{,}8 \times 10^{-6}$	6,33	4,11	1,54
210107_at	CLCA1	$19{,}3 \times 10^{-3}$	6,76	4,38	1,54
228658_at	MIAT	$1{,}3 \times 10^{-3}$	7,70	4,92	1,56
222101_s_at	DCHS1	$131{,}8 \times 10^{-6}$	9,24	5,83	1,58
219855_at	NUDT11	$4{,}6 \times 10^{-3}$	8,70	5,49	1,58
224367_at	BEX2	$4{,}5 \times 10^{-3}$	9,81	6,02	1,63
230896_at	BEND4	$2{,}9 \times 10^{-3}$	7,20	4,40	1,64
222281_s_at	NA	$131{,}8 \times 10^{-6}$	7,72	4,69	1,65
205347_s_at	TMSB15A	$14{,}8 \times 10^{-3}$	10,15	6,12	1,66
209706_at	NKX3-1	$1{,}3 \times 10^{-3}$	9,26	5,53	1,68
211549_s_at	HPGD	$195{,}4 \times 10^{-6}$	8,50	5,03	1,69
207250_at	SIX6	$1{,}3 \times 10^{-3}$	8,42	4,96	1,70
206135_at	ST18	$4{,}6 \times 10^{-3}$	7,07	4,00	1,77
203914_x_at	HPGD	$478{,}4 \times 10^{-6}$	9,43	5,30	1,78
225864_at	FAM84B	$1{,}2 \times 10^{-6}$	8,30	4,54	1,83
211548_s_at	HPGD	$1{,}3 \times 10^{-3}$	8,74	4,68	1,87
203913_s_at	HPGD	$131{,}8 \times 10^{-6}$	9,13	4,63	1,97

SECONDARY TOPIC I:

"FUNCTIONAL PROTEIN NETWORK ACTIVATION MAPPING REVEALS NEW POTENTIAL MOLECULAR DRUG TARGETS FOR POOR PROGNOSIS PEDIATRIC BCP-ALL"

3.1 B-CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA (BCP-ALL)

The majority of ALLs are of B-cell lineage origin in both children (>80%) and adults (>75%) [7][160]. In B cell precursor ALL (BCP-ALL) blast cells are regarded as malignant counterparts of normal B-cell precursors.

Current approaches of risk assessment rely on a number of key clinical and laboratory findings such as the initial leukocyte count, age at diagnosis and early treatment response. Children aged 1-9 years have a better outcome than infants and adolescents. Leukocyte count is a continuous variable, with decreasing counts conferring a better outcome [161]. However, the most informative prognostic factor is the response to early treatment, as determined by measurements of the rate of clearance of leukemic cells from the blood or bone marrow. This estimate of minimal residual disease accounts for the drug sensitivity or resistance of leukemic cells and the pharmacodynamics of the drugs, which is affected by the pharmacogenetics of the host [162]. Flow cytometric profiling of aberrant immunophenotype markers and polymerase chain reaction amplification of fusion transcripts or antigen-receptor genes, which are at least 100-fold more sensitive than conventional morphological determinants, have allowed minimal residual disease to be detected at very low levels (< 0.01%). This provides a useful means

to identify patients at very low or high risk of relapse and to adapt treatment intensity to stratified protocols (Standard, Medium, High Risk) [163].

But in spite of treatment and risk stratification improvement, 20% of children with ALL ultimately relapse, and cure rate after relapse reaches only 25% to 40%. It is therefore important to identify prognostic markers that allow to predict the outcome of patients, analyzing samples characteristics at diagnosis. Moreover, a relevant role is assigned to the understanding of leukemic cell biology and its aberrant features compared to normal condition.

Beside MRD stratification, childhood acute leukemias have long been the best characterized malignancies from a genetic viewpoint. The most significant impact for risk stratification for treatment are t(9;22)(q34;q11)/BCR-ABL1 and rearrangements of the MLL gene. In particular this applies to t(4;11)(q21;q23)/MLL-AF4. The prognosis of the other MLL transolcations may become significant in the future, particularly among infants [164]. The detection of these two abnormalities provides the basic criteria for the classification of high risk groups, which is applied in all American and European protocols.

Other significant structural abnormalities include t(12;21)(p13;q22)/ETV6-RUNX1 fusion, as well as t(1;19)(q23;p13.3)/TCF3-PBX1 fusion. However, these are not used in risk stratification on all protocols. The ETV6-RUNX1 fusion occurs in approximately 25% of younger children with BCP-ALL and these patients have an extremely good prognosis. Among patients with TCF3 rearrangements, those with TCF3- PBX1 were originally regarded as poor risk on some treatment protocols, but on the current therapeutic protocol they are classified as standard risk [165][166]. In contrast the rare variant, t(17;19)(q22;p13)/HLF-TCF3 fusion, has a dismal outcome on all therapies [167].

Intrachromosomal amplification of chromosome 21 (iAMP21) consists of an abnormal chromosome 21 with highly variable morphology between patients and with a common amplified region that in all cases includes the RUNX1 gene [168][169][170]. This abnormality was originally described as poor risk factor [171][172][173], although the outcome at this moment has been shown to be protocol dependent [174].

Numerical cytogenetic abnormalities are also reported to be important for outcome prediction: high hyperdiploidy (51–65 chromosomes) [175], near-haploidy (24–29 chromosomes) and low hypodiploidy (31–39 chromosomes) [176][177]. High hyperdiploidy accounts for approximately 30% of childhood BCP-ALL and is characterized by the gain of specific chromosomes. It is associated with a good prognosis in children. Near-haploidy and low hypodiploidy are rare, comprising <1% each of childhood ALL, and both are linked to a poor outcome and are used to stratify patients as high risk.

Translocations involving IGH@ at 14q32 are emerging as a significant subgroup in childhood ALL [178][179][180][181]. It is of interest that they occur more frequently in adolescents and, although numbers are small, they appear to have an inferior outcome.

Recurring genomic aberrations that define leukemia subtypes are important in leukaemia initiation [182], but alone are insufficient to generate a full leukaemic phenotype, indicating that cooperating oncogenic lesions are present in leukaemia specimens. Although additional mutations have been identified in a subset of cases, the full complement of cooperating lesions and their distribution within the genetic subtypes of ALL remain to be defined.

The most notable observation was the identification of genomic alterations in genes that regulate B-lymphocyte differentiation in about 40% of B-progenitor ALL cases. The most common targets of these genetic alterations are EBF1, PAX5 and IKZF1 that have central roles in the development of normal B cells [183]. New identified deletions include IKZF3, LEF1, TCF3 and BLNK, encoding regulators of B-cell development. Other deleted loci are BTG1, recently associated with glucocorticoids resistance *in vitro* [184], and ERG, reported to occur exclusively in a subgroup of BCP-ALL associate to a favourable outcome [185].

Recently, a cryptic translocation, t(X;14)(p22;q32) or t(Y;14)(p11;q32), involving IGH@ and CRLF2 in the pseudoautosomal region (PAR1) of the sex chromosomes, and a deletion within PAR1, giving rise to the P2RY8-CRLF2 fusion, have been reported [186][187][188]. These genomic alterations lead to

overexpression of CRLF2, which has been defined as a novel, significant abnormality in BCP-ALL. CRLF2 alterations, including activating mutations of the CRLF2 receptor itself, are associated with activating JAK mutations resulting in constitutive activation of the JAK-STAT signalling pathway [188][189][190]. Activation of this pathway has been associated with a worse prognosis in adults and children with BCP-ALL [7][191] and has been highlighted as an important candidate pathway for targeted therapy.

In the last years, the molecular understanding of this pediatric disease, together with more effective chemotherapy protocols and best patients stratification, has led significant progresses in cure of BCP-ALL patients. Nevertheless, new informations are needed to understand cases that still experience resistance to treatment and encounter relapse. The study of the functional proteome in BCP-ALL could highlight new biomarkers and molecular targets useful for the development of new therapeutic strategies.

BCP-ALL characteristics are summarized in Table 3.1.

Table 3.1: Characteristics and clinical outcomes of selected subtypes of childhood BCP-ALL. (Modified from Pui et al., 2011 [4])

Subtype	Frequency (%)	Clinical Implication	Estimated 5-Year Event-Free Survival (%)
B-cell precursor			
Hyperdiploidy > 50	20-30	Excellent prognosis with antimetabolite-based therapy	85-95
t(12;21)(p13;q22) *ETV6-RUNX1*	15-25	Expression of myeloid-associated antigens CD13 and CD33; excellent prognosis with intensive asparaginase therapy	80-95
Trisomies 4 and 10	20-25	Excellent prognosis with antimetabolite therapy	85-90
t(1;19)(q23;p13) *TCF3-PBX1*	2-6	Increased incidence in blacks; excellent prognosis with high-dose methotrexate treatment; increased risk of CNS relapse in some studies	80-85
Intrachromosomal amplification of chromosome 21	2-3	More common in older children and adolescents; poor prognosis; benefit from intensive induction and early re-intensification therapy	30-40
t(4;11)(q21;q23) *MLL-AF4*	1-2	Poor prognosis and predominance in infancy, especially those < 6 months of age; overexpression of *FLT3*	30-40
t(9;22)(q34;q11.2) *BCR-ABL1*	2-4	Imatinib plus intensive chemotherapy improve early treatment outcome	80-90 at 3 years
t(8;14)(q23;q32.3)	2	Favorable prognosis with short-term intensive therapy with high-dose methotrexate, cytarabine, and cyclophosphamide	75-85
Hypodiploidy < 44 chromosomes	1-2	Poor prognosis	35-40
CRLF2 overexpression	6-7	Poor prognosis; common in patients with Down syndrome (55%)	?
Cooperation mutations			
B-cell precursor			
IKZF1 deletions/mutations	15-30	Poor prognosis; resistant to asparaginase and daunorubicin	50-55
JAK mutations	?2-5	Predominance in high-risk patients; *JAK2* mutations in 20% of Down syndrome cases; potentially responsive to *JAK2* inhibitors	~60

Abbreviation: ALL, acute lymphoblastic leukemia.

3.2 "FUNCTIONAL PROTEIN NETWORK ACTIVATION MAPPING REVEALS NEW POTENTIAL MOLECULAR DRUG TARGETS FOR POOR PROGNOSIS PEDIATRIC BCP-ALL"

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PUBLISHED IN PLoS One. 2010 Oct 21;5(10).

3.2.1 Abstract

Background. In spite of leukemia therapy improvements obtained over the last decades, therapy is not yet effective in all cases. Current approaches in Acute Lymphoblastic Leukemia (ALL) research focus on identifying new molecular targets to improve outcome for patients with a dismal prognosis. In this light phosphoproteomics seems to hold great promise for the identification of proteins suitable for targeted therapy.

Methodology/Principal Findings. We employed Reverse Phase Protein Microarrays to identify aberrantly activated proteins in 118 pediatric B-cell precursor (BCP)-ALL patients. Signal transduction pathways were assayed for activation/expression status of 92 key signalling proteins. We observed an increased activation/expression of several pathways involved in cell proliferation in poor clinical prognosis patients. MLL-rearranged tumours revealed BCL-2 hyperphosphorylation through AMPK activation, which indicates that AMPK could provide a functional role in inhibiting apoptosis in MLL-rearranged patients, and could be considered as a new potential therapeutic target. Second, in patients with poor clinical response to prednisone we observed the up-modulation of LCK activity with respect to patients with good response. This tyrosine-kinase can be down-modulated with clinically used inhibitors, thus modulating LCK activity could be considered for further studies as a new additional therapy for prednisone-resistant patients. Further we also found an association between high

levels of CYCLIN E and relapse incidence. Moreover, CYCLIN E is more expressed in early relapsed patients, who usually show an unfavourable prognosis.

Conclusions/Significance. We conclude that functional protein pathway activation mapping revealed specific deranged signalling networks in BCP-ALL that could be potentially modulated to produce a better clinical outcome for patients resistant to standard-of-care therapies.

3.2.2 Introduction

Acute Lymphoblastic Leukemia (ALL) is the most common form of pediatric cancer with a worldwide incidence of about 1–4.75 per 100000 persons [192]. Remarkable progress has been made in treatment of childhood ALL but therapy is not yet effective in all cases. Current research interest focuses on identifying new specific molecular drug targets for new patient-tailored approaches that can improve therapy efficacy and reduce toxicity. Knowledge of deregulation of cell signalling pathways in cancer that regulate and control cell proliferation, differentiation, survival and death forms the basis for understanding tumour progression. Recent publications have placed elucidation of protein signalling pathways at the central point in the effective treatment of cancer [193][194]. Pathway activation and function is controlled by post-translational modifications, mainly by phosphorylation, underpinned by ongoing activity of protein kinases and phosphatases. Consequently, functional pathway mapping technology that can directly measure the activation state of hundreds of proteins in signalling transduction pathways (STPs), can hold great promise for the identification of altered STPs in tumour cells. Such efforts promise to potentially provide new targets for rational, molecular-targeted drug design and could identify cancer patients that may benefit from the use of specific targeted inhibitors [54][195]. Protein activation status can not be directly analyzed through gene expression profiling, since post-translational modifications, such as phosphorylation, are not predictable from gene expression levels. Here, Reverse Phase Protein Microarray (RPMA) technology had been used to profile the activation state of 92 key molecules in a cohort of 118 newly diagnosed precursor pediatric BCP-ALL

patients, in order to identify and map pathway activation changes associated with clinical characteristics. This innovative technique can measure the activation levels/phosphorylation of large numbers of signalling proteins at once from small clinical samples in a very reproducible, precise, sensitive and high-throughput manner. The RPMA format immobilizes in spots dozens of different patient samples on one array and each array is then incubated with a specific antibody, thus a single endpoint is measured and directly compared across multiple samples without introduction of experimental variability. This cutting-edge technology has already been applied with success to profile the cellular STPs activity in several cancers [54][57][69][70][195][196]. We observed an increase or decrease in activation/phosphorylation state of signalling proteins within specific protein networks in clinical poor prognosis patients cohorts. In particular, here we show the inhibition of the LCK kinase in Prednisone Good Responder (PGR) patients, and a hyperactivated pathway in the MLL-rearranged cohort of patients that leads to BCL-2 activation through LKB1 and AMPK phosphorylation. Moreover, we found a correlation between CYCLIN E expression and Relapse Free Survival (RFS) rates: patients who show high levels of CYCLIN E expression have a more elevated probability to relapse. These new informations on pediatric BCP-ALL activated protein patterns provided by phosphoproteomic analyses with RPMA will be the start for future functional studies with specific protein inhibitors, in order to point out new drugs for patient tailored therapies.

3.2.3 Results

3.2.3.1 Correlation between Protein Expression and Clinical Characteristics

We first searched for correlation between protein expression/activation and patients clinical characteristics. In particular we considered the followings: age (1-9 years vs > 9 years), sex, white blood cell count (WBC > vs < of 50 x 109/L), DNA index (1-1.15 vs ≥ 1.16), chromosomal translocations (non-translocated, t(9;22), t(12;21), t(1;19) and MLL rearrangements), Minimal Residual Disease (Low Risk, Medium Risk, High Risk), immunophenotype (Prepre-B, Pre-B, CALL, Prepre-B/CALL) and prednisone response through Wilcoxon tests or two-

sample Welch t-tests implemented in multtest package. No correlation was found between protein expression/activation and age, sex, WBC, DNA index, Minimal Residual Disease (MRD) and immunophenotype (data not shown), but we observed differentially activated/expressed proteins in MLL-rearranged vs non-translocated and in Prednisone Good Responder (PGR) vs Prednisone Poor Responder (PPR) patients comparisons.

3.2.3.2 AMPK Pathway is Hyperactivated in MLL-rearranged Patients

We compared primary leukemia samples isolated from 8 MLL-rearranged patients (5 with t(4;11), 2 with t(9;11), and one with t(11;19)) with 36 patients without known genomic aberrancies. Statistical analysis (Wilcoxon test with Benjamini-Hochberg multiplicity corrections) revealed different expression or activation of 9 proteins between the MLL-rearranged patients and the non-translocated ones. Our results show that 4 proteins were statistically significantly elevated in the MLL-rearranged patients group: CYCLIN E (p= 0.02425), ANNEXIN 2 (p= 0.02910), AMPKβ (S108) (p= 0.02910) and AMPKα (S485) (p= 0.03686). Furthermore a set of 3 more proteins, eNOS/NOS III (S116 – corresponding to S114 in human), LKB1 (S428) and BCL-2 (S70), was found to be differentially activated in the MLL-rearranged cohort using a Global Test analysis (p=0.003) (Fig. 3.1-A). These 3 proteins are all known members of the AMPK pathway (see Discussion) and, along with the findings that AMPK itself was activated in the MLL-rearranged cohort, form the basis of a comprehensive pathway derangement in MLL-rearranged patients (presented schematically in Fig. 3.1-B). We thus identified a singular MLL-specific hyperactivated pathway that through AMPK phosphorylation leads to the activation of BCL-2. A heatmap was generated to highlight the relationships between clustering and protein expression levels (Fig. 3.1-C). RPMA results were validated by Western Blot in an independent set of patients (Fig. 3.2-A). Of note, total forms of the AMPK pathway proteins do not show substantial differences between MLL-rearranged and non-translocated patients (Fig. 3.2-B), corroborating the observation that the

higher phosphorylation levels of the proteins in the AMPK pathway are the peculiar molecular derangement characteristic of MLL-rearranged BCP-ALL.

Additionally, we asked whether it was possible to identify a difference in the gene expression levels of the same AMPK-related genes that we identified through RPMA analysis among MLL-rearranged and non-translocated patients. We analyzed the gene expression profiles of 29 MLL-rearranged and 41 non-translocated pediatric BCP-ALL patients. All the patients included in this analysis were part of a larger cohort of samples analyzed by gene expression profiling during the international "Microarray Innovation in Leukemia" (MILE) study [153].

The unsupervised analysis with the 15 probe sets corresponding to Lkb1, Ampkα and β, eNos and Bcl-2 genes was not able to accurately separate MLL-rearranged and non-translocated patients (Fig. S3.1-A). Moreover, when performing a comparative analysis between MLL-rearranged and non-translocated patients using the 15 probe sets of the AMPK-related genes, only one probe set (PRKAA 214917_at, corresponding to Ampkα) resulted to be differentially expressed between the two groups with a fold change more than 2.0 (Fig. S3.2-B). It is of note that this probe set resulted to be upregulated in the non-translocated

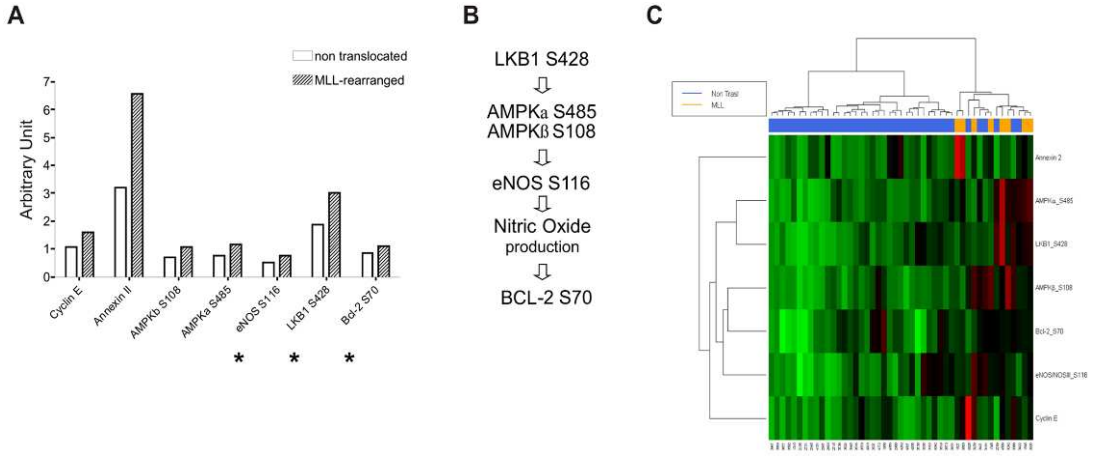

Fig. 3.1:Hyperactivation of the AMPK pathway in MLL-rearranged patients.
(A) Histogram of proteins, that are part of the AMPK pathway, found to be differentially activated (Wilcoxon testwith Benjamini-Hochberg multiplicity correction, $p<0.05$) between the MLL- and non-translocated cohorts. * indicates proteins that are part of the AMPK pathway, but that did reach statistical significance using Global Test analysis ($p= 0.003$). (B) Scheme of the AMPK pathway. (C) Heatmap with hierarchical clustering. The heatmap was generated with R Project using the proteins differentially expressed/phosphorylated in the "MLL-rearranged patients" group vs "non-translocated patients" group comparison. MLL-rearranged patients are highlighted in orange.

group of patients.

In order to verify the relationships kinase-substrate within the AMPK pathway, we treated the two MLL-rearranged cell lines SEM and RS4;11 with the commercial AMPK inhibitor Compound C. As shown in Fig. 3.2-C, after AMPK inhibition the activation levels of AMPKα and β and all the downstream targets

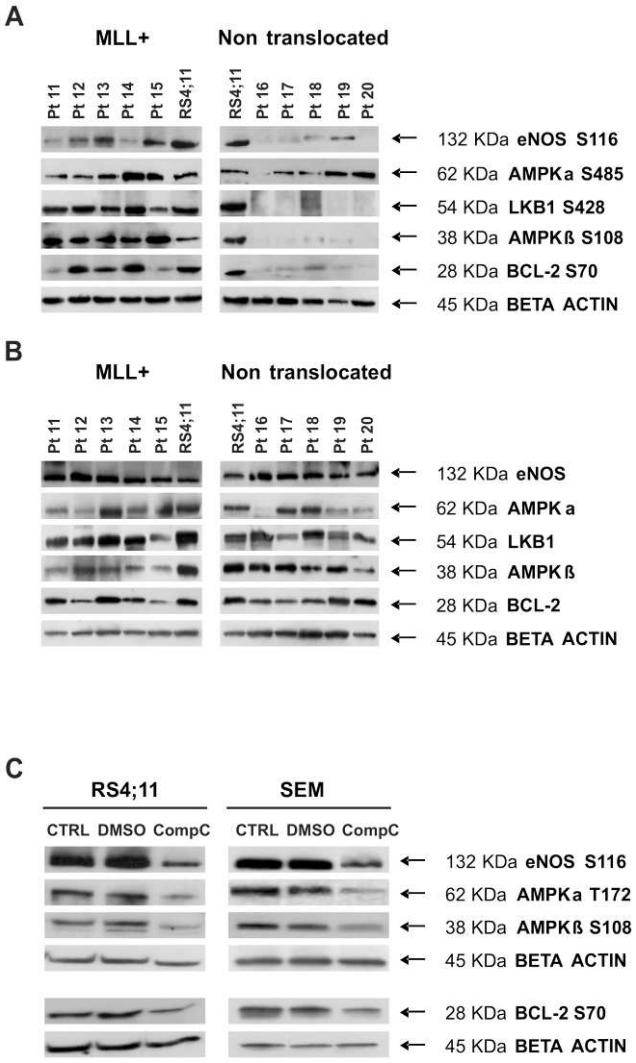

Fig. 3.2:Validation of RPMA results through Western Blot.
(A) Hyperactivation of the AMPK pathway in MLL-rearranged patients vs non-translocated ones (independent sets of pediatric BCP-ALL at diagnosis: patients 11-15 are MLL-rearranged -all MLL-AF4-, patients 16-20 are non-translocated). RS4;11 cell lysate was used as positive control for antibody staining. (B) Total forms of the AMPK pathway proteins in previously described patients: 11-15 are MLL-rearranged and 16-20 are non-rearranged. RS4;11 cell lysate was used as positive control for antibody staining. There are no substantial differences on total protein form levels between MLL-rearranged and non-translocated patients. (C) AMPK pathway inhibition after Compound C treatment. RS4;11 and SEM cells (both MLL-rearranged) were treated with the AMPK inhibitor Compound C 8µM for 48 hours. Phosphorylation of AMPK pathway proteins was evaluated through WB in control, DMSO treated and Compound C treated cells.

are markedly decreased, confirming the functional link between these proteins. In addition, apoptosis is induced in MLL-rearranged cell lines after Compound C treatment (LC50 8µM, 48h), while other two human non-translocated BCP-ALL cell lines are insensitive to AMPK inhibition (data not shown).

Our data provide evidence that in MLL-rearranged patients a number of directly connected kinase-substrates are activated, and this can contribute to the chemotherapy resistance observed in these patients.

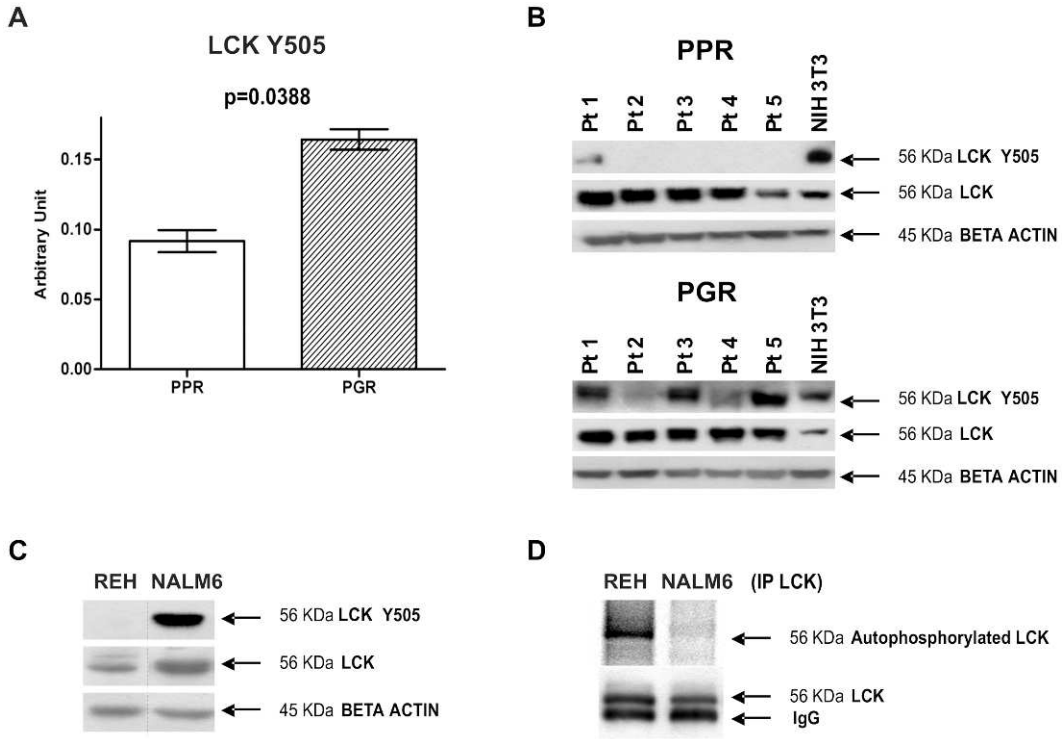

Fig. 3.3:Hyperactivation of LCK in PPR patients.
(A) LCK Y505 expression measured with RPMA is higher in Prednisone Good Responder (PGR) (0.164 ± 0.007) than in Prednisone Poor Responder (PPR) (0.092 ± 0.008) patients (two-sample Welch t-statistics -unequal variances- with Bonferroni multiplicity corrections p=0.0388). (B) LCK hyperphosphorylation at Y505 in PGR patients was confirmed by Western Blot in an independent set of specimens (pediatric BCP-ALL at diagnosis: patients 1-5 are Prednisone Good Responders, patients 6-10 are Prednisone Poor Responders). NIH3T3 commercial cell lysate (BD Biosciences) was used as positive control for antibody staining. Expression of the total form of LCK does not differ between the patients. (C) LCK activation state in the cell lines chosen as in vitro model. REH cell lines are a model for PPR patients, while NALM6 are a model for PPR patients. The LCK activation state was evaluated through WB. (D) In vitro LCK autophosphorylation assay. LCK was immunoprecipitated from REH and NALM6 cells, and then incubated in a phosphorylation mixture at 30°C for 40min. Autophosphorylation of LCK was analyzed by digital autoradiography on Cyclone Plus (upper gel), and by WB with anti-LCK antibody to assess the LCK amount (lower gel).

3.2.3.3 LCK Activity is Down-Modulated in Prednisone Good Responder Patients

We compared the phosphoproteomic profile of 9 Prednisone Poor Responder (PPR) patients vs 109 Prednisone Good Responder (PGR) patients. Statistical analyses (two-sample Welch t-statistics -unequal variances- with Bonferroni multiplicity corrections) revealed that in PGR patients the inhibited form of the kinase LCK (LCK phosphorylated at Y505) was higher than in PPR patients ($p=0.0388$) (Fig. 3.3-A). We confirmed the overexpression of the inhibited form of LCK in PGR patients by Western Blot in an independent set of patients (Fig. 3.3-B).

In order to provide biochemical evidence of a hyperactivation state of LCK in PPR cells compared to PGR cells, we performed radioactive autophosphorylation assay of LCK. To this purpose we selected REH cells as a model for PPR patients and NALM6 cells as a model for PGR patients [197]. We first confirmed that LCK is more phosphorylated in Y505 in NALM6 cells than in REH cells (Fig. 3.3-C): when similar amounts of total LCK were analyzed, the LCK Y505 phosphorylation was evident almost exclusively in NALM6 cells. As expected, on the contrary, the autophosphorylation activity of immunoprecipitated LCK was significantly higher in REH cells (Fig. 3.3-D).

This tyrosine-kinase, important in the regulation of growth and differentiation of eukaryotic cells, resulted to be more activated in cells that do not respond to glucocorticoids treatment, and thus activation of LCK could be considered as a putative marker for prednisone resistance.

3.2.3.4 CYCLIN E is Upregulated in Relapsed Patients

We performed a Relapse Free Survival (RFS) analysis considering patients included in AIEOP LLA 88, 91, 95 and 2000 therapy protocols in order to identify proteins related to a more aggressive disease. Relapse Cumulative Incidence were obtained through Kaplan-Meier estimates and the difference between two curves was assessed by log rank test. CYCLIN E, that regulates cell cycle steps critical for growth control, had been demonstrated to be overexpressed in many malignant

tumors (for a review see Möröy et al. [198]), and in particular it could be useful to assess malignancy of blasts in adult B- and T-ALL [199]. Similarly to van Rhenen et al. [200] we thus searched for a threshold value for CYCLIN E that resulted in the largest difference in survival between the two groups defined by that threshold. We considered 10 equispaced cutoff values between the first quartile and the third quartile. A threshold was deemed valid only if the difference in the Relapse Cumulative Incidence curves was statistically significant (CYCLIN E threshold 1.228, log rank test after Bonferroni corrections p=0.000075) (Fig. 3.4-A). We found that patients with CYCLIN E levels higher than 1.228 have a higher probability to relapse: 15 of 28 (53%) patients with CYCLIN E higher than 1.228 relapsed, while among the 84 patients with CYCLIN E lower than 1.228 only 14 (17%) relapsed. We confirmed RPMA CYCLIN E levels by Western Blot (Fig. 3.4-B). Moreover, we did observe that 6 of 7 MLL-rearranged patients and 3 of 3 patients with t(9;22) are in the high CYCLIN E expression group. Furthermore, 16 of 19 patients MRD-Standard Risk have low levels of CYCLIN E, and 4 of 5 patients MRD-High Risk have high levels of CYCLIN E. In the groups determined by the above threshold we searched for differences in age, sex, immunophenotype, chromosomal translocations, DNA index, WBC count, prednisone response and MRD. From this multivariate analysis, CYCLIN E expression resulted independent from all other considered variables. We also compared CYCLIN E expression in early vs late relapsed patients. By definition, early relapsed patients are those patients who suffered of a relapse within 6 months after stopping front-line treatment, and they show a worst prognosis with respect to late relapsed patients [201]. CYCLIN E resulted significantly higher in early relapsed patients (Wilcoxon test p=0.002) (Fig. 3.4-C). Because CYCLIN E in conjunction with its kinase subunit CDK2 regulates essential processes for entering into the S-phase, we also looked if differences between relapsed and non-relapsed patients could be associated to CYCLIN E together with CDK2. After Global Test analysis, the two proteins resulted to be correlated (p=0.009395) in our patients cohort. These observations taken together strongly suggest that CYCLIN E could be considered as a marker for the aggressiveness of the disease.

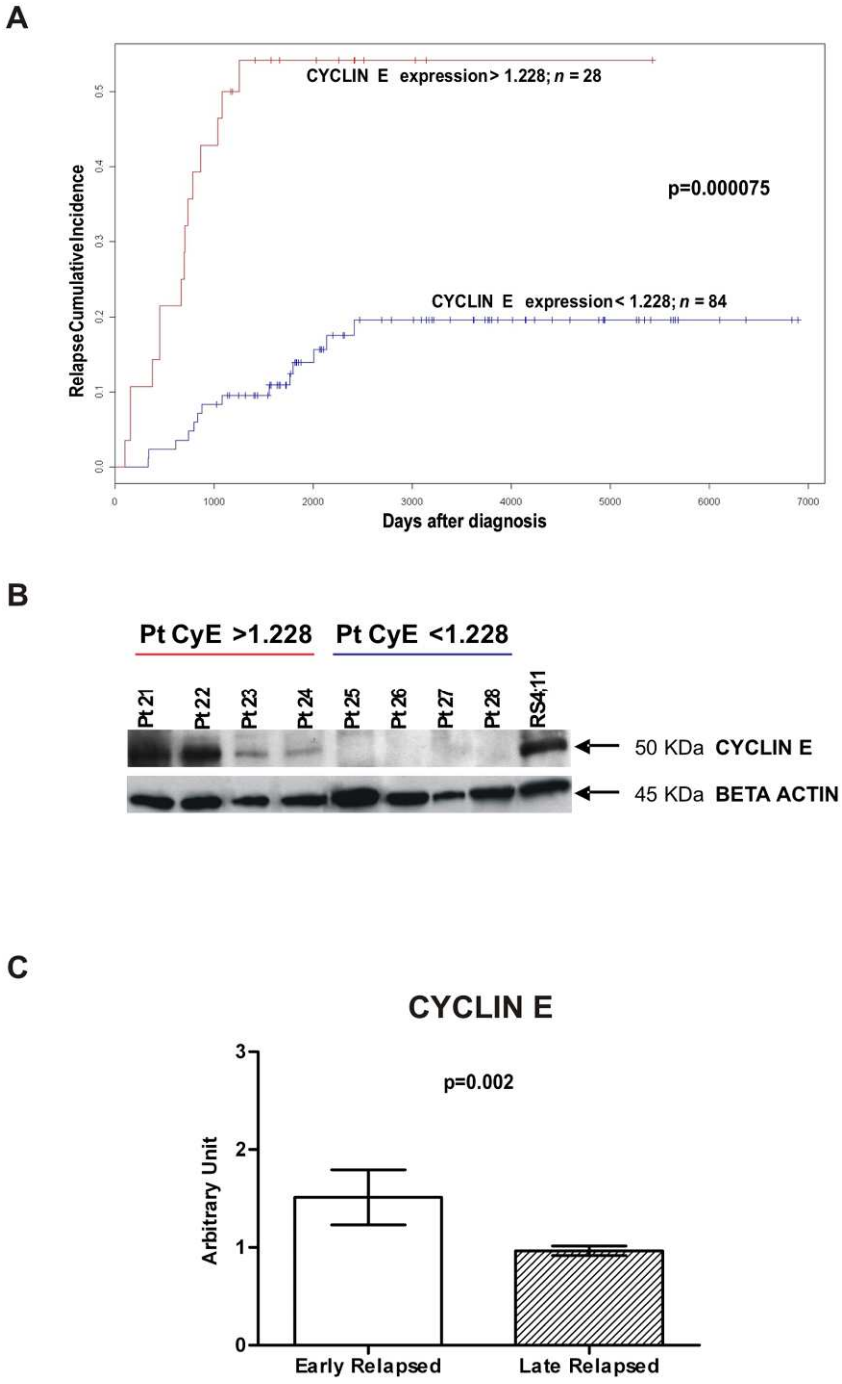

Fig. 3.4:CYCLIN E expression and Relapse Free Survival.
(A) Relapse Cumulative Incidence comparison between patients with CYCLIN E expression levels < 1.228 (n=84, blue line) and patients with CYCLIN E levels > 1.228 (n=28, red line). Patients with elevated CYCLIN E levels have a higher probability to relapse (CYCLIN E threshold 1.228, log rank test after Bonferroni corrections p=0.000075). (B) RPMA results validation with Western Blot. Patients 21-24 showed high levels of CYCLIN E after RPMA analysis: 1.974, 1.896, 1.537, 1.506 respectively. Patients 25-28 showed low levels of CYCLIN E after RPMA analysis: 0.463, 0.470, 0.298, 0.292 respectively. RS4;11 cell lysate was used as positive control for antibody staining. (C) Early relapsed patients (relapsed within 910 days from diagnosis) show higher levels of CYCLIN E expression with respect to late relapsed patients (Wilcoxon test p=0.002).

3.2.4 Discussion

Despite dramatic improvements in leukemia therapy over the last decades, about 20% of patients does not achieve a stable complete disease remission. In the past few months, new findings using genome-wide mutational analysis have placed central importance on protein pathway deregulation as the principal driving force in many human malignancies [193][194]. We have originated the RPMA technology to directly quantitatively measure protein pathway function and activation to broadly map signal transduction networks and profile human cancers in order to identify groups of patients with specific molecular aberrations and to identify new targets for therapy. In this study we used the RPMA global pathway mapping technique in pediatric BCP-ALL patients to map the pathway activation status of 92 different phosphorylated and total proteins of key signalling “hubs” known to be involved in human tumorigenesis and metastasis. Correlations of signalling activation with clinical response/follow-up and known genetic information (e.g. gene rearrangements), enabled us to identify new protein pathway biomarkers that, when validated in larger clinical sets, could be used for patient stratification and targeted therapy trials.

Our first main finding concerns infants with MLL (Mixed Lineage Leukemia) rearrangements. MLL translocations are present in about 6% of pediatric leukaemia patients, especially in infants with ALL where about 75% of patients are MLL-rearranged, and their presence predicts early relapse and poor prognosis (event-free survival of 28–45%) [161][202]. We identified a singular MLL-specific hyperactivated pathway that through AMPK phosphorylation leads to the activation of BCL-2, a well known anti-apoptotic regulator crucial for chemotherapy resistance already found to be over-expressed both at mRNA and protein levels in MLL-rearranged leukemias [203]. This pathway derangements appear to emanate from LKB1, a serine/threonine kinase that has been shown to phosphorylate AMPK [204]. AMP-activated protein kinase (AMPK) is a serine/threonine kinase that acts as a cellular fuel sensor, activated under conditions that deplete ATP and elevate AMP levels such as metabolic or environmental stresses. AMPK is known to activate endothelial nitric oxide

synthase (eNOS) [205][206] and thus to stimulate Nitric Oxide (NO) production. NO is a multifunctional transcellular messenger that can play a dual role in cancer with both pro- and anti-apoptotic effects [207][208]. Interestingly, a prominent NO production has been observed in undifferentiated tumours [209] such as the MLL-rearranged leukemias. In addition, it has been reported that NO can prevent apoptosis by elevating BCL-2 expression both at mRNA and protein levels in B-lymphocytes [210][211]. We showed that treatment of two MLL-rearranged cell lines with the AMPK inhibitor Compound C not only brings to AMPK deactivation, but also of its described downstream targets. This confirms the kinase-substrate relationship between these proteins, and highlights the essential contribute of AMPK in sustaining the activation of this pathway. Activation of AMPK had been studied in several tumour types because it usually leads to antitumor effects [212], but we found AMPK phosphorylation to be at the highest relative levels in the MLL-rearranged subgroup. We speculate that the role of AMPK pathway in leukemias and other hematological tumours may be different from solid epithelial malignancies, as already reported by Baumann et al. [213] in multiple myeloma cells. Indeed, it will be of primary interest to inhibit AMPK, and thus BCL-2, activity using commercially available AMPK inhibitors in order to more fully elucidate the functional role of AMPK activation in MLL-rearranged patients, and to evaluate AMPK as a potential new therapeutic target for this specific subgroup of patients. The mRNA results, indicating that the AMPK pathway genes are not upregulated in MLL-rearranged patients, sustain the importance to deepen with protein activation analyses in order to better define disease-related disorders in cellular metabolism, and thus to identify new molecular drug targets.

One of the strongest independent predictive factors for therapy outcome in childhood ALL is the response to initial prednisone treatment. Prednisone response is considered a backbone in Berlin-Frankfurt-Münster (BFM)-oriented protocols, and is defined by the number of peripheral leukemic blasts on day 8 of therapy [214][215]. The threshold value for distinction between good and poor response is 1000 blasts/µl. In trial ALL-BFM 90, PGR patients showed a 6-years

Event Free Survival (EFS) of 82%, while for PPR patients this was only 34% [216]. Here, we observed that in PGR patients LCK is less activated than in PPR patients (LCK Y505, p=0.0388), and we exploited the autophosphorylation assay of LCK in order to biochemically confirm its higher activity in PPR cells. LCK is a non receptor protein-tyrosine kinase of the Src oncogene family mostly expressed in T cells, where plays an essential role in activation and development, and in some B cells and other cancer tissues [217][218]. Its activity is primarily regulated by phosphorylation, catalyzed by the kinase CSK, at the tyrosine residue Y505, located near the C-terminus, leading to protein deactivation [219]. In the future, if ongoing validation continues to implicate LCK activation as a predictive marker for prednisone resistance, we will investigate the causal significance of this and possibly implicate this molecule as a therapeutic target that could modulate prednisone response mechanisms. Very interestingly, inhibition of LCK through treatment with kinase inhibitors currently used in clinical practice for other indications such as BMS-354825 (Dasatinib) and STI-571 (Imatinib) [220] had already been demonstrated to be able to induce apoptosis in human T cells. Thus, it will be of interest to establish if LCK inhibitors could be useful as a possible additional support in BCP-ALL PPR patients treatment.

Our third main result is that high levels of CYCLIN E expression are indicator of a more aggressive disease. Patients who show elevated CYCLIN E expression have a higher probability to relapse. CYCLIN E had been demonstrated to be overexpressed in many malignant tumors (for a review see Möröy et al. [198]), and, in particular, Scuderi et al. [199] reported that BCP-ALL blasts of adult patients had high CYCLIN E levels and relapsed samples displayed additional accumulation of the protein. CYCLIN E regulates cell cycle progression through the restriction point R, at the end of the G1-phase, to allow cells to enter S-phase inducing S-phase specific genes. The restriction point R has been recognized to be critical for growth control and thus also for the prevention of unrestricted cell proliferation, malignant transformation and tumorigenesis. We found a cutoff value of CYCLIN E expression able to distinguish patients who have a higher probability to relapse (53% vs 17%), and this is independent from all other

clinical and molecular variables. Interestingly, the elevated CYCLIN E expression group is anyhow enriched in poor prognosis patients (MLL-rearranged, t(9;22) translocated and MRD-High Risk), and CYCLIN E is more expressed in early relapsed (within 910 days from the diagnosis) patients who have a worst prognosis [201] with respect to late relapsed patients. This indicates that CYCLIN E expression could correlate with the malignant potential of the cells, and thus could be considered as a marker of the aggressiveness of the disease and a new therapeutic target in pediatric BCP-ALL.

This study emphasizes the importance of protein pathway activation mapping analysis of clinical specimens as a route for discovery of functional derangement that may be functional, causative agents of the cancer. Proteins related to proliferation and survival such as LCK, AMPK and CYCLIN E were found to be hyperactivated or overexpressed in poor prognosis patients with BCP-ALL, and could represent new molecular drug targets in pediatric B-ALL. When further validated in functional studies, specific kinase inhibitors that target AMPK pathway, LCK-mediated signalling and CYCLIN E activation could be evaluated in prospective clinical trials whereby patients who are in need of better therapeutic options could be selected and stratified for targeted therapeutics tailored to the molecular defect.

3.2.5 Materials and Methods

3.2.5.1 Ethics Statement

The study was approved by the Ethical Committee board of the University of Padova, the Padova Academic Hospital and the Italian Association of Pediatric Onco-Hematology (AIEOP). Patient's parents or their legal guardians provided written informed consent following the tenets of the Declaration of Helsinki.

3.2.5.2 Patients

Bone marrow samples from 118 children with newly diagnosed BCP-ALL were analyzed. Diagnosis was made according to standard cytomorphology, cytochemistry and immunophenotypic criteria [152]. The study was approved by

the local ethics committees and informed consent was obtained for all patients. Samples were collected at the Pediatric Oncohematology Laboratory (Padua, Italy), between 1990 and 2006 and stored in the BioBank in liquid nitrogen in FCS+DMSO. Bone marrow mononuclear cells from patients were separated by Ficoll-Hypaque technique (Pharmacia, Uppsala, Sweden) and frozen within 3hours after collection. The whole blood blast percentage for all samples was between 70% and 98%. Patients molecular and clinical characteristics are resumed in Table S3.1.

3.2.5.3 Cell lines

Human leukemia cell lines SEM, RS4;11, REH and NALM6 were purchased from DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). SEM and RS4;11 cell lines derive from BCP-ALLs carrying the t(4;11) MLL-AF4 translocation. REH cell line derives from a BCP-ALL carrying the t(12;21) TEL-AML1 translocation. NALM6 cells derive from a BCP-ALL without recurrent chromosomal translocations. Cells were cultured in RPMI 1640 (Biochrom AG, Berlin, Germany) with 10% FCS, penicillin (100U/ml) and streptomycin (100µg/ml), and maintained at 37°C in a humidified atmosphere with 5% CO_2.

3.2.5.4 Reverse Phase Protein Microarrays

Cell Lysis. Cells were washed with ice–cold PBS 1X and lysed on ice for 20minutes in an appropriate lysis buffer: TPER Reagent (Pierce, Rockford, IL), 300mM NaCl, 1mM Na orthovanadate, 200mM PEFABLOC (AEBSF) (Roche, Basel, Switzerland), 1ug/mL Aprotinin (Sigma, St. Louis, MO), 5mg/mL Pepstatin A (Sigma), 1mg/mL Leupeptin (Sigma). Cell lysates were then cleared by centrifugation and supernatants were collected and assayed for protein concentration with the Coomassie Protein Assay Reagent Kit (Pierce). Cell lysates were diluted to 1mg/ml in a mixture of 2x Tris-Glycine SDS Sample Buffer (Invitrogen Life Technologies, Carlsbad, CA) plus 5% of β-Mercaptoethanol. Lysates were stored at -80°C and boiled 8 minutes immediately prior to arraying.

RPMA Printing. Lysates were loaded into a 384-well plate and serially diluted with lysis buffer into four-point dilution curves ranging from undiluted-1:8. As positive controls for antibody staining we added also 3 commercial cell line lysates: A431+EGF, Hela+Pervanadate and Jurkat Apoptotic cell lysates (BD Biosciences, Franklin Lakes, NJ). We divided the 118 samples in 2 set of arrays, thus 59 and 59 samples were printed in duplicate in each array set onto nitrocellulose-coated slides (FAST slides, Whatman Schleicher & Schuell, Florham Park, NJ) with the 2470 Arrayer (Aushon BioSystems, Burlington, MA). On each set of arrays also the above mentioned cell lines and 2 bridge samples were printed for antibody signal normalization between the 2 sets. Printed slides were stored dessicated (Drierite, Sigma) at -20°C until use.

RPMA Staining. Selected slides were stained with Sypro Ruby (Invitrogen) according to the manufacturer's instruction, in order to estimate the total protein amount of each printed sample. Before antibody staining the arrays were treated with ReBlot Plus Mild Antibody Stripping Solution (Chemicon, Temecula, CA) 1X for 15minutes at room temperature, rinsed 2 times for 5minutes in PBS 1X, and then blocked for 1hour at room temperature in blocking solution (2gr I-Block - Tropix, Bedford, MA - and 0.1% Tween-20 in 1l of PBS 1X). Blocked arrays were stained with antibodies on an automated slide stainer (Dako Autostainer Plus, Dako Cytomation, Carpinteria, CA) using the CSA kit (Dako Cytomation) as described previously [6]. Slides were air dried and scanned on a PowerLook 1000 flatbed scanner (UMAX, Dallas, TX) at 600dpi. For an example of antibody-stained slides please see Fig. S3.2.

For the complete list of the 92 stained antibodies with RPMA, please see Table S3.2. Each antibody was previously subjected to extensive validation for single band specificity by Western Blot (WB). For phospho-specific antibodies, each antibody was checked for specificity using cell extracts with and without appropriate ligand induction. The 92 antibodies used in this study were carefully selected based on both their extensive validation for specificity as well as detecting key signalling molecules known for their involvement in motility, invasion, pro-survival, and growth factor signalling.

Image Analysis. The TIF images of antibody- and Sypro Ruby-stained slides were analyzed using Microvigene Software (VigeneTech Inc, Boston, MA) to extract numeric intensity values from the arrays images. Briefly this software, specifically developed for RPMA analysis, localizes the spots and subtracts the local background, calculating pixel intensity for each spot. The software calculates these values in the antibody-stained slides, the corresponding negative control slides (secondary antibody alone) and the total protein slide. Then, for each sample, the signal of the negative control array is subtracted from the antibody slide signal and then the resulting value is normalized to the total protein value, to ensure that intensity values were not dependent on changes in concentration of printed lysate. The data values were normalized to either one of the bridging cases to facilitate comparison of sample values between paired arrays stained with the same antibody. The data processing generates normalized single value for each protein measured for each sample.

3.2.5.5 Western Blot

The following antibodies were used for WB at the concentrations reported in parentheses. Primary antibodies: anti-AMPKα (23A3) (1:1000), anti-phospho-AMPKα S485 (1:1000), anti-AMPKß1 (1:1000), anti-phospho-AMPKα T172 (1:1000), anti-AMPKß1 (1:1000), anti-phospho-AMPKß S108 (1:1000), anti-BCL-2 (1:1000), anti-phospho-BCL-2 S70 (1:1000), anti-LCK (L22B1) (1:1000), anti-LCK Y505 (1:500), anti-β-ACTIN (1:1000) (all from Cell Signaling Technology, Inc, Danvers, MA), CYCLIN E (BD Biosciences) (1:500), anti-eNOS/NOS III CT (1:1000), anti-phospho-eNOS/NOS III S116 (both from Upstate – Millipore, Billerica, MA) (1:1000). Secondary antibodies: HRP-Goat anti-rabbit and anti-mouse IgG-conjugate (Zymed Laboratories, Inc., South San Francisco, CA) (1:50000). Total cell lysates were analyzed by SDS-PAGE under reducing conditions, and transferred to a nitrocellulose sheet (Hybond-P, GE Healthcare, Chalfont St. Giles, UK) following standard methods. Membranes were saturated for 3hours with 2% Amersham ECL Advance Blocking Reagent (GE Healthcare), primary antibodies were incubated overnight at 4°C and

secondary antibodies for 1hour at room temperature. The immunoreactivity was determined by an enhanced chemiluminescent reaction (Amersham ECL ADVANCE Western Blotting Detection Kit, GE Healthcare). For the stripping, membranes were incubated for 30minutes in constant rocking in a solution 25mM Glycin, 1% SDS and pH2, then washed in T-PBS 1X and resaturated.

3.2.5.6 AMPK inhibition in MLL-rearranged cell lines

AMPK was specifically inhibited in SEM and RS4;11 cell lines using Compound C (Calbiochem, Darmstadt, Germany) 8µM for 48 hours. Proteins were extracted from treated and control cells as described for RPMA. Of note, in order to carefully determine the activation level of AMPKα, in these experiments we measured the phosphorylation of the main activation site that is the T172.

3.2.5.7 Immunoprecipitation and in vitro kinase assay

REH and NALM6 cell lines were chosen as in vitro model for PPR and PGR patients respectively. Cells were lysed in a buffer containing 20mM Tris-HCl, pH 7.5, 150mM NaCl, 2mM EDTA, 2mM EGTA, 0.5% (v/v) Triton X-100, 2mM dithiothreitol, protease inhibitor cocktail Complete (Roche), 10mM NaF, 1µM okadaic acid, 1mM Na orthovanadate. LCK was immunoprecipitated with limiting amount (20ng) of anti-LCK 3A5 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), for 4h at 4°C, followed by addition of protein A-Sepharose (Sigma). 100µM Na vanadate was present throughout the incubations. Immunoprecipitates were washed once with NET buffer (50mM Tris–HCl pH 8.0, 150mM NaCl, 5mM EDTA, 0.05% (v/v) Nonidet P-40, 2mg/ml bovine serum albumin) and twice with 50mM Tris–HCl pH 7.5, then incubated at 30°C for 40min with a phosphorylation mixture containing 50mM Tris-HCl, pH 7.5, 10mM MgCl2, 10mM MnCl2 10µM [γ-33P]ATP (specific radioactivity ~5000cpm/pmol) and 50µM Na orthovanadate, in a total volume of 20ml. Samples were then boiled for 5min, loaded onto 11% SDS–PAGE followed by blotting to Immobilon-P membranes (Millipore); autophosphorylation of LCK was analyzed by digital autoradiography on Cyclone Plus (PerkinElmer, Waltham,

MA) to detect the radioactivity and by WB with anti-LCK to assess the LCK amount.

3.2.5.8 Statistical Analysis

Statistical analyses were performed with R. Identification of activated proteins was obtained through Wilcoxon tests or two-sample Welch t-tests implemented in multtest package [221]. Pearson's chi-squared test was used for clinical variables. Pathways were identified using global test [222]. P-values were corrected for multiplicity using Benjamini-Hochberg method to control false discovery rate or with Bonferroni method to control family wise error rate; therefore the reported p-values are adjusted p-values. Survival curves were obtained through Kaplan-Meier estimates and the difference between two curves was assessed by log rank test. Finally, a heatmap was generated to highlight the relationships between clustering and protein expression levels.

3.2.6 Supporting information

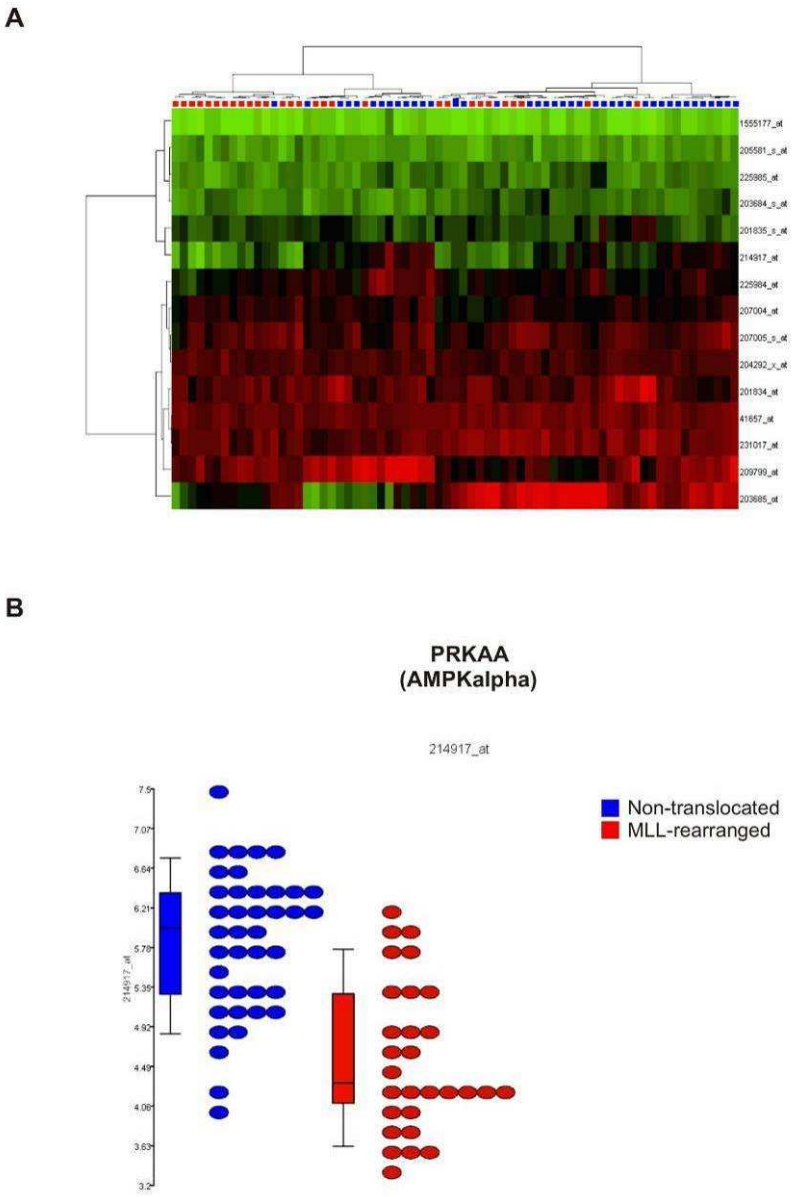

Fig. S3.1:AMPK-related genes are not upregulated in MLL-rearranged patients.
(A) Heatmap generated with Partek Genomics Suite software using the probe sets corresponding to Lkb1, Ampkα and β, eNos and Bcl-2 genes. The unsupervised analysis is not able to accurately separate MLL-rearranged and non-translocated patients. MLL-patients are highlighted in red. (B) Dot Plot representing the raw expression data of PRKAA probe set (214917_at). The comparative analysis, performed with Partek Genomics Suite software, between MLL-rearranged (red) and non-translocated (blue) patients, using the 15 probe sets of the AMPK-related genes, shows only one probe set (PRKAA, corresponding to Ampkα) differentially expressed between the two groups with a fold change more than 2.0. AMPKα results upregulated in the non-translocated patients. Each dot represents a patient, and the boxes represent the median expression values for each group of specimens. The expression values on y axis are reported on log2 scale.

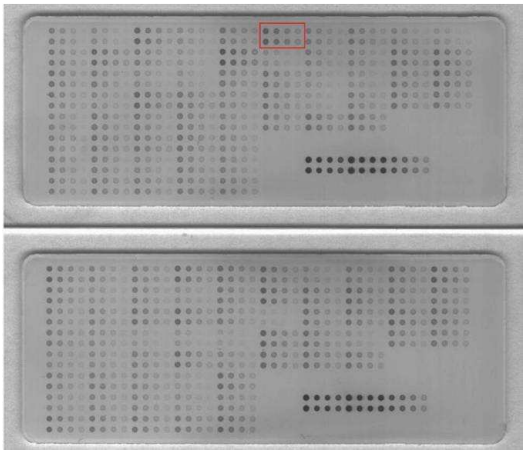

Fig. S3.2:Example of RPMA stained slides.
Slides in the picture are stained with LCK Y505 antibody. Each patient lysate was printed in a four-point dilution curve ranging from undiluted to 1:8 in duplicate (an example is framed in red). Samples were divided in 2 set of arrays, thus 59 and 59 samples were printed in duplicate in each array set onto nitrocellulose-coated slides. As positive controls for antibody staining, in the right lower part of the slides we added 3 commercial cell line lysates: A431+EGF, HeLa+Pervanadate and Jurkat Apoptotic cell lysates. On each set of arrays the above mentioned cell lines and 2 bridge samples were used for antibody signal normalization between the 2 sets.

Table S3.1:Patients clinical and molecular characteristics.

Characteristic	***Number (%)***
Sex	
Male	62 (52.5)
Female	56 (47.5)
Age (years)	
<1	6 (5.1)
1-9	92 (78.0)
>9	20 (16.9)
AIEOP[a] therapy protocol	
LLA[b] 88	1 (0.8)
LLA 91	16 (13.6)
LLA 95	35 (29.6)
LLA 2000	62 (52.5)
Interfant	4 (3.4)
Immunophenotype	
Prepre-B	10 (8.5)
Pre-B	30 (25.4)
CALL	76 (64.4)
Prepre-B/CALL	2 (1.7)
WBC Count	
>50 x 10^9/L	34 (28.8)
<50 x 10^9/L	84 (71.2)
DNA Index	
1-1.15	88 (74.6)
≥1.16	19 (16.1)
ND[c]	11 (9.3)
Prednisone Response	
Good	109 (92.4)
Poor	9 (7.6)
Chromosome Translocation	
Neg[d]	36 (30.5)
t(12;21)	21 (17.8)
t(1;19)	7 (5.9)
t(9;22)	3 (2.5)
MLL rearrangements	8 (6.8)
ND	43 (36.5)
MRD (only AIEOP LLA 2000 patients)	
Standard Risk	19 (30.6)
Medium Risk	38 (61.3)
High Risk	5 (8.1)
Outcome	
Relapsed	32 (27.1)
Dead	15 (12.7)
Complete Remission	71 (60.2)

[a]AIEOP= Italian Association of Pediatric Oncohematology.
[b]LLA= Acute Lymphoblastic Leukemia.
[c]ND= No Data.
[d]Neg= negative for MLL-rearrangements, 12;21, 1;19 and 9;22.

Table S3.2: Primary antibodies for RPMA staining.

Antibody	***Company***	***RPMA Dilution***
14_3_3	Upstate Biotechnology Inc.	1:20000
4EBP1 S65	Cell Signaling Technology, Inc.	1:100
AKT S473	Cell Signaling Technology, Inc	1:100
AKT T308	Cell Signaling Technology, Inc	1:100
AMPKα S485	Cell Signaling Technology, Inc	1:50
AMPKβ S108	Cell Signaling Technology, Inc	1:50
Annexin 1	BD Transduction Laboratories	1:5000
Annexin 2	BD Transduction Laboratories	1:500
Ask1 S83	Cell Signaling Technology, Inc	1:50
ATF2 T71	Cell Signaling Technology, Inc	1:100
Bad S112	Cell Signaling Technology, Inc	1:100
Bad S136	Cell Signaling Technology, Inc	1:50
Bad S155	Cell Signaling Technology, Inc	1:100
Bak	Cell Signaling Technology, Inc	1:50
Bax	Cell Signaling Technology, Inc	1:250
Bcl-2 S70	Cell Signaling Technology, Inc	1:250
Bcl-xL	Cell Signaling Technology, Inc	1:500
Cdk2	Cell Signaling Technology, Inc	1:100
Chk1 S345	Cell Signaling Technology, Inc	1:50
Chk2 S33/35	Cell Signaling Technology, Inc	1:50
c-Kit Y703	Zymed Laboratories, Inc.	1:500
Cleaved Casp 3 D175	Cell Signaling Technology, Inc	1:50
Cleaved Casp 6 D162	Cell Signaling Technology, Inc	1:50
Cleaved Casp 7 D198	Cell Signaling Technology, Inc	1:1000
Cleaved Casp 9 D315	Cell Signaling Technology, Inc	1:250
Cleaved Casp 9 D330	Cell Signaling Technology, Inc	1:50
Cleaved PARP D214	Cell Signaling Technology, Inc	1:100
CREB S133	Cell Signaling Technology, Inc	1:100
Cyclin A	Cell Signaling Technology, Inc	1:20
Cyclin D1	BD Transduction Laboratories	1:20
Cyclin E	BD Transduction Laboratories	1:100
EGFR Y1068	Cell Signaling Technology, Inc	1:50
EGFR Y1148	Cell Signaling Technology, Inc	1:200
EGFR Y992	Cell Signaling Technology, Inc	1:100
eIF4G S1108	Cell Signaling Technology, Inc	1:1000
Elk1 S383	Cell Signaling Technology, Inc	1:100
eNOS-eNOSIII S116	Upstate Biotechnology Inc.	1:500
ERK1/2 T202/Y204	Cell Signaling Technology, Inc	1:2000
Estrogen Rec α S118	Cell Signaling Technology, Inc	1:1000
FADD S194	Cell Signaling Technology, Inc	1:250
FAK Y397	BD Transduction Laboratories	1:50
FHKR S256	Cell Signaling Technology, Inc	1:50
FHKR/L1 T24/T32	Cell Signaling Technology, Inc	1:200
Grb2	Cell Signaling Technology, Inc	1:1000
GSK3αβ S21-9	Cell Signaling Technology, Inc	1:100
GSK3αβ S279/216	BioSource	1:500
HIF1α	BD Transduction Laboratories	1:20
HSP70	Stressgen	1:200
IKBα S32/36	BD Transduction Laboratories	1:50
IRS-1 S612	Cell Signaling Technology, Inc	1:100
Jak1 Y1022/1023	Cell Signaling Technology, Inc	1:100

Lck Y505	Cell Signaling Technology, Inc	1:500
LKB1 S428	Cell Signaling Technology, Inc	1:100
MARCKS S152/156	Cell Signaling Technology, Inc	1:50
MEK1/2 S217/221	Cell Signaling Technology, Inc	1:400
MSK1 S360	Cell Signaling Technology, Inc	1:50
mTOR S2448	Cell Signaling Technology, Inc	1:100
p27 Kip1	BD Transduction Laboratories	1:100
p38 MAPK T180/Y182	Cell Signaling Technology, Inc	1:100
p70 S6 T412	Upstate Biotechnology Inc.	1:500
p70 S6 T389	Cell Signaling Technology, Inc	1:20
p90 RSK S380	Cell Signaling Technology, Inc	1:400
PAK1 S199/204-PAK2 S192/197	Cell Signaling Technology, Inc	1:50
PDGFRβ Y716	Upstate Biotechnology Inc.	1:250
PDK1 S241	Cell Signaling Technology, Inc	1:200
PKA C T197	Cell Signaling Technology, Inc	1:200
PKCα S657	Upstate Biotechnology Inc.	1:2000
PKCαβII T638/641	Cell Signaling Technology, Inc	1:100
PKCδ T505	Cell Signaling Technology, Inc	1:100
PKCθ T538	Cell Signaling Technology, Inc	1:100
PKCζ/λ T410/403	Cell Signaling Technology, Inc	1:50
PRAS40 T246	BioSource	1:3000
PTEN S389	Cell Signaling Technology, Inc	1:500
PYK2 Y408	Cell Signaling Technology, Inc	1:200
A-Raf S299	Cell Signaling Technology, Inc	1:50
B-Raf S445	Cell Signaling Technology, Inc	1:50
C-Raf S338	Cell Signaling Technology, Inc	1:200
Ras-GRF1 S916	Cell Signaling Technology, Inc	1:50
S6 Ribosomal Protein S235/236	Cell Signaling Technology, Inc	1:200
SAPK/JNK T183/Y182	Cell Signaling Technology, Inc	1:200
SGK1	Upstate Biotechnology Inc.	1:200
Shc Y317	Cell Signaling Technology, Inc	1:250
SHIP Y1020	Cell Signaling Technology, Inc	1:50
Smad2 S465/467	Cell Signaling Technology, Inc	1:250
Src Y416	Cell Signaling Technology, Inc	1:100
Src Y527	Cell Signaling Technology, Inc	1:500
STAT1 Y701	Cell Signaling Technology, Inc	1:50
STAT3 S727	Cell Signaling Technology, Inc	1:200
STAT3 Y705	Upstate Biotechnology Inc.	1:100
STAT5 Y694	Cell Signaling Technology, Inc	1:50
STAT6 Y641	Cell Signaling Technology, Inc	1:100
Tuberin-TSC2 Y157	Cell Signaling Technology, Inc	1:50

3.2.7 Acknowledgements

We thank Dr. V. Calvert and Dr. J. Wulfkuhle for helpful discussion, Dr. E. Giarin for her precious help with the BioBank management, Dr. A. Leszl for providing cytogenetics data, Dr. S. Varotto for providing patients clinical informations, Dr. S. Bresolin for gene expression analyses and L. Marchioretto for technical support.

3.2.8 Author Contributions

Conceived and designed the experiments: BA, VE, GTK, MR, EFP, LAL, GB. Performed the experiments: BA, AJV, GM, LG, GM, MS, LT. Analyzed the data: MG, BA. Interpreted the data: BA, VE, GTK, MR, EFP, LAL, GB. Wrote the paper: BA, MG, GTK, MR, EFP. Final approval: VE, MR, EFP, LAL, GTK, GB, RDM.

3.3 "AMPK INHIBITION PROMOTES APOPTOSIS IN MLL-REARRANGED PEDIATRIC B-ACUTE LYMPHOBLASTIC LEUKEMIA CELLS"

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PAPER IN SUBMISSION

In pediatric B-Cell Precursor-Acute Lymphoblastic Leukemia (BCP-ALL) patients with MLL-rearrangements the serine/threonine kinase AMPK and its downstream effectors, including eNOS and BCL-2, are hyperactivated. In this paper we investigate the role of activated AMPK in supporting MLL-rearranged leukemia cell survival and we evaluate AMPK as a new druggable target.

In order to study the functional role of the AMPK pathway in the survival of MLL-rearranged cells, we used four BCP-ALL cell lines. RS4;11 and SEM cell lines are BCP-ALL carrying the (4;11) MLL-AF4 translocation. AMPK is activated in these cells, similarly to what is observed in primary cells from MLL-rearranged patients. On the other hand, AMPK activation is not detected in MHH-CALL-2 and MHH-CALL-4, which are non-translocated BCP-ALL cell lines (Accordi Plos One 2010). We thus used these cell lines to study the effects of AMPK inhibition on BCP-ALL cell survival. AMPK activity was inhibited using the commercial inhibitor Compound C, resulting first in a cell cycle block at the G2/M phase (Fig. 3.5) and then in massive apoptosis selectively in MLL-

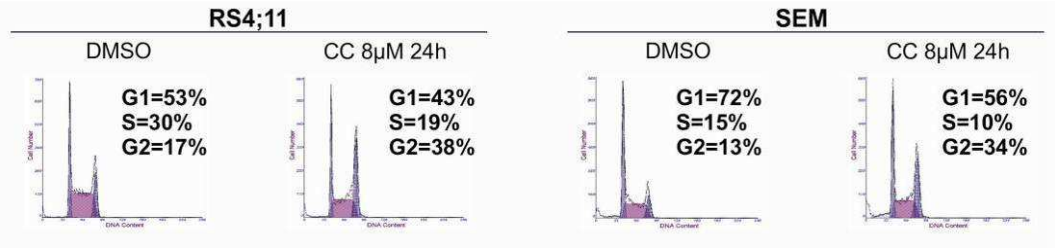
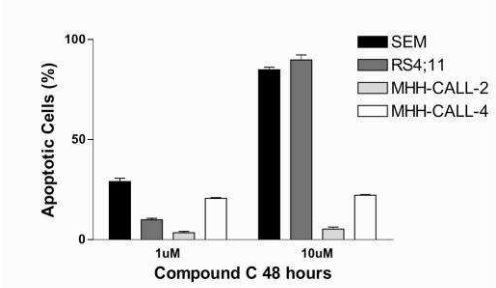


Fig. 3.5:. **Cell cycle analysis of treated (CC -Compound C- 8µM, 24 hours) MLL-rearranged cell lines.** Samples were analyzed by flow cytometry and cell cycle analyses were performed using Multicycle Wincycle software. The percentage of each phase of the cell cycle (G1, S, G2/M) was calculated. Control cells are cultured with DMSO.

rearranged cells. The LC50 is 7.5µM and 8.5µM for SEM and RS4;11 respectively, whereas it increases to 37.5µM for MHH-CALL-2 and 31.4µM for MHH-CALL-4 (Fig. 3.6).



	LC_{50} (µM) 48H
SEM	7.5
RS4;11	8.5
MHH-CALL-2	37.5
MHH-CALL-4	31.4

Fig. 3.6:. **Cells were treated with Compound C at different concentrations ranging from 0.01µM up to 100µM for different times (6-96 hours).** Cell viability was determined by flow cytometry with Annexin V-PI staining. DMSO treated cells viability was set to 100%. Results represent Mean of 3 independent experiments ± SEM. The LC_{50} (Lethal $Concentration_{50}$= compound concentration required to induce cell mortality by 50%) was calculated by plotting the data as a logarithmic function of (x) when viability was 50%.

We also observed that Compound C-induced apoptosis associates with mitochondrial membrane depolarization (disruption of JC-1 aggregates, Fig. 3.7), reactive oxygen species production (hydroethidine HE production, Fig. 3.8), Cytochrome c release and Caspase-9, -7 and PARP cleavage (Fig. 3.9), indicating intrinsic apoptosis pathway activation.

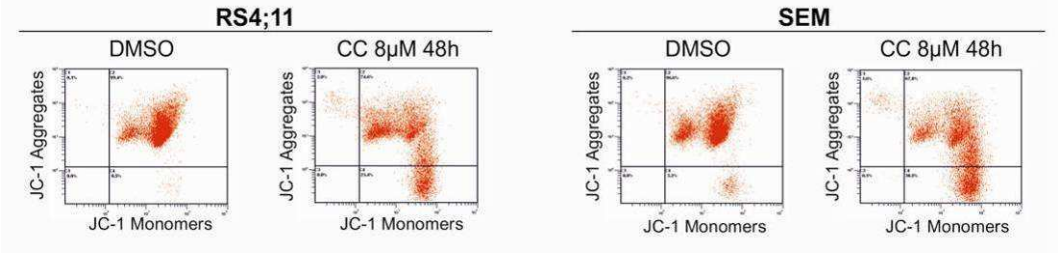


Fig. 3.7:. **MLL-rearranged cells were treated with Compound C (CC 8µM, 48 hours) and the depolarization of the mitochondrial transmembrane potential was monitored by the fluorescent dye JC-1**. The method is based on the ability of this fluorescent probe to enter selectively into the mitochondria, and its colour changes reversibly from green to orange as membrane potential increases. This property is due to the reversible formation of JC-1 aggregates upon membrane polarization. Aggregation causes a shift in the emitted light from 530nm (emission by JC-1 monomers) to 590nm (emission by JC-1 aggregates) following excitation at 490nm.

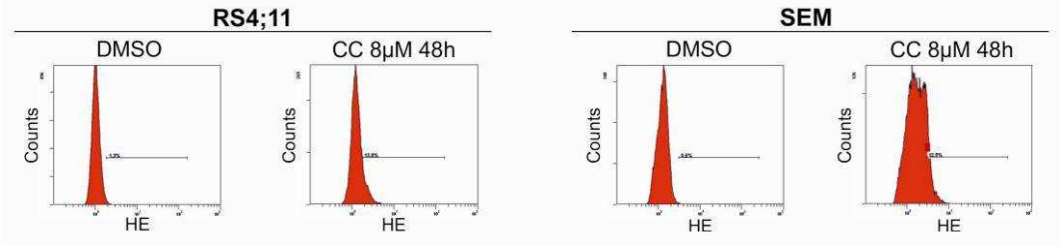


Fig. 3.8:. **MLL-rearranged cells were treated with Compound C (CC 8µM, 48 hours) and the mitochondrial production of reactive oxygen species was investigated.** The fluorescence indicator hydroethidine (HE), that is oxidized by superoxide anion into ethidium ion which emits red fluorescence, was measured.

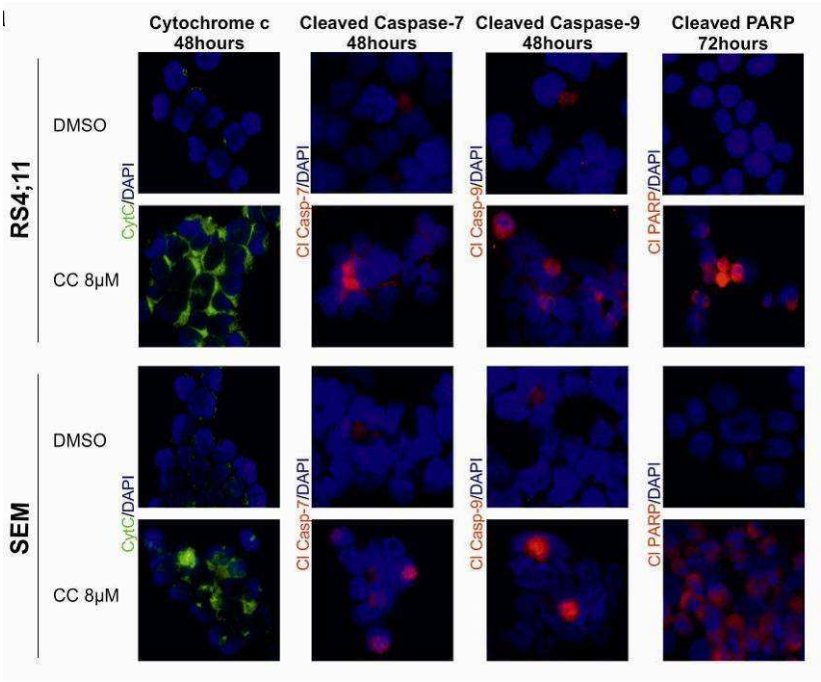


Fig. 3.9:. **Apoptotic proteins expression was determined by immunofluorescence in MLL-rearranged cells after Compound C (CC 8µM, 48 and 72 hours) treatment.** Slides were analyzed by epifluorescence with a ViCo (Video Confocal) microscope (Nikon Eclipse 80i). Images were acquired with ImageProPlus (Media Cybernetics) and collected at magnification 60X.

We also treated 8 primary BCP-ALL cultures (4 MLL-rearranged and 4 non-translocated) with Compound C in order to determine its effects on patients blast cells. MLL-rearranged patients responded very well to AMPK inhibition also at low Compound C concentrations (1µM and 5µM), undergoing much more apoptosis than non-translocated patients (t test, p=0.005 1µM 24h, p=0.008 5µM 24h, p=0.03 1µM 48h, p=0.04 5µM 48h) (Fig. 3.10). Western Blot analyses

demonstrated the dephosphorylation of AMPK, eNOS and BCL-2 after treatment both in cell lines and primary samples.

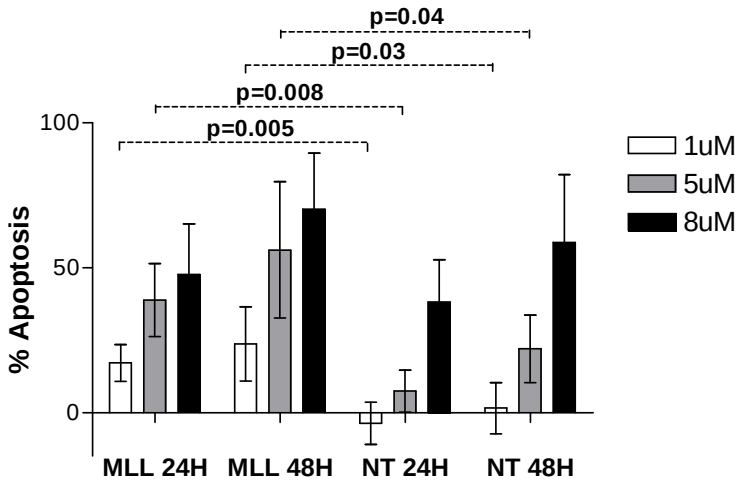


Fig. 3.10:. **Primary BCP-ALL cultures (4 MLL-rearranged and 4 non-translocated) treated with Compound C in order to determine its effects on patients blast cells.** MLL-rearranged patients response to AMPK inhibition at low Compound C concentrations (1µM and 5µM), undergoing much more apoptosis than non-translocated patients (t test, p=0.005 1µM 24h, p=0.008 5µM 24h, p=0.03 1µM 48h, p=0.04 5µM 48h).

With our research we provide new insights into the role of AMPK in cancer and in particular in pediatric BCP-ALL. AMPK activation is required for MLL-rearranged cell survival and its inhibition is able to induce cell death. For these reasons AMPK can be considered as a new drug target in MLL-rearranged leukemias and kinase inhibitors targeting AMPK should be further studied in order to make new therapeutic options available for these poor prognosis patients.

SECONDARY TOPIC II:

"HIGH RISK ACUTE LYMPHOBLASTIC LEUKEMIA WITH RAPID NOD/SCID ENGRAFTMENT IS CHARACTERIZED BY HIGH PROTEIN EXPRESSION OF CYCLIN B, BETA-CATENIN, ANNEXIN I AND DECREASED PKC ALPHA ACTIVATION"

In collaboration with Felix Seyfried and Dr. Lüder Hinrich Meyer, Ulm University, Germany.

Felix Seyfried, Benedetta Accordi, Manon Queudeville, Sarah Mirjam Eckhoff, ***Gloria Milani****, Luisa Galla, Marco Giordan, Johann Kraus, Giuseppe Basso, Hans Kestler, Geertruy te Kronnie, Klaus-Michael Debatin, Lüder Hinrich Meyer*

53rd Annual Meeting of the American Society of Hematology (San Diego, California - Dec. 10th-13th 2011) (Poster)
BLOOD, Journal of the American Society of Hematology. Vol. 118, N0 21:p633. PUBLICATION NUMBER: 1457

4.1 INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common form of pediatric cancer. Despite therapy efforts have achieved cure rates of above 80%, about every fifth patient encounters relapse, which is associated with poor outcome. Novel methods are needed to identify new prognostic markers that might refer to therapeutic targets for patients with an unfavorable prognosis.

Meyer et al. [223] recently investigated the engraftment properties and impact on patient outcome of 50 pediatric acute lymphoblastic leukemia samples transplanted into NOD/SCID mice. Time to leukemia (TTL) was determined for each patient sample engrafted as weeks from transplant to overt leukemia. Short

TTL was strongly associated with high risk for early relapse, identifying an independent prognostic factor. Rapid leukemia engraftment of primary ALL cells (short time to leukemia/ TTL^{short}) in a NOD/SCID/huALL mouse xenotransplant model is thus indicative of early relapse (Meyer *et al*., Cancer Cell 2011).

4.2 AIM OF THE STUDY – SECONDARY TOPIC II -

In this study we aimed to identify differently expressed and activated proteins in xenograft ALL of distinct engraftment subgroups employing a Reverse Phase Protein Arrays (RPPA) strategy. This is to point out deregulated signalling pathways responsible for the different time of engrafment and thus for the early onset of relapse in patients.

4.3 MATERIALS AND METHODS

Levels of total or activated proteins were analyzed in patients derived xenograft B cell precursor (BCP-) ALL samples (N=16) characterized with respect to their NOD/SCID engraftment phenotype.

The whole proteome of each sample was immobilized on nitrocellulose coated glass slides and overall protein expression or phosphorylation of 51 key signalling molecules was detected by incubation with the corresponding specific antibodies. Protein expression and/or activation was quantified and compared between the distinct engraftment subgroups. RPPA findings were validated by Western Blot analyses.

4.4 RESULTS

In this study, 7 of 16 xenografted patient derived leukemia samples led to rapid engraftment (< 10 weeks, TTL^{short}) with an inferior relapse free survival of the corresponding patients in contrast to 9 patients with TTL^{long} phenotype (Fig. 4.1).

Comparison of the protein expression data (Shrinkage t-test, $P < .05$, fold change ≥ 1.5) in the TTL subgroups identified differentially expressed proteins. CYCLIN B was up-regulated in leukemia samples with rapid NOD/SCID/huALL growth (TTL^{short}/early relapse phenotype) and correlates negatively to TTL (Fig.

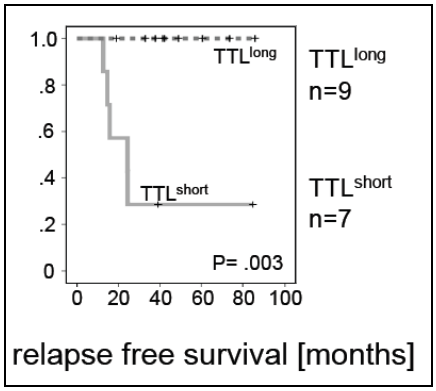


Fig. 4.1: TTL groups. Inferior relapse free survival of TTL^{short} patients.

4.2). Beta-CATENIN was found to be over-expressed in TTL^{short} and protein levels were reversely correlated to TTL (Fig. 4.3). Additionally, ANNEXIN I showed high protein levels in TTL^{short} (Fig. 4.4).

In contrast, higher levels of PKC alpha activation were identified in the TTL^{long}-group (Fig. 4.5). RPPA findings were confirmed by Western blotting, showing similar result.

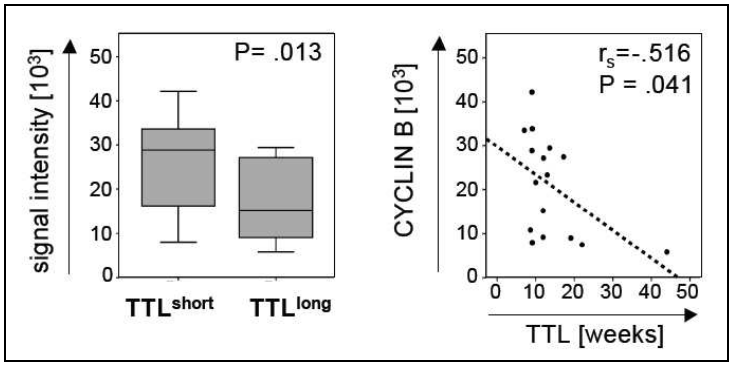


Fig. 4.2: CYCLIN B up-regulation in leukemic samples with rapid NOD/SCID/huALL growth.

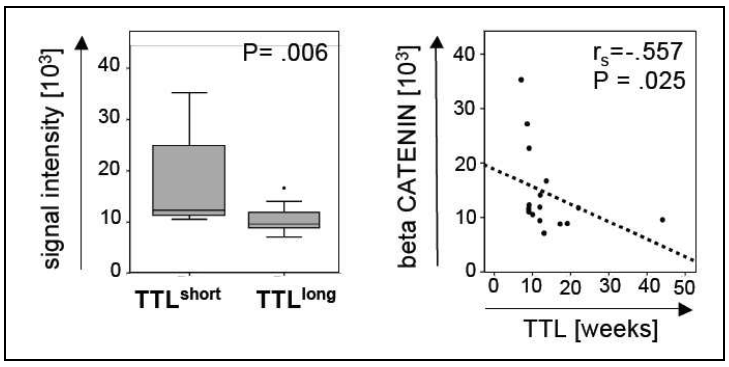


Fig. 4.3: beta-CATENIN overexpression in TTL^{short}.

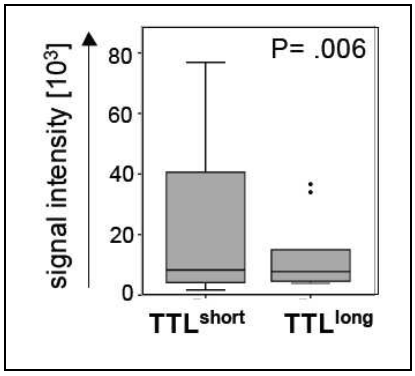


Fig. 4.4: Annexin I high levels in TTL^{short}.

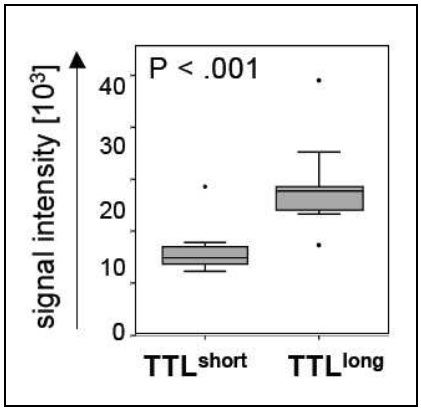


Fig. 4.5: PKC alpha downregulation in TTL^{short}.

4.5 DISCUSSION

This study revealed differentially expressed proteins in prognostic subgroups of pediatric BCP-ALL patients with distinct clinical outcomes. CYCLIN B is a positive regulator of the cell cycle and highly expressed in the G2/M phase. Consistent with its pro-proliferative function, it was up-regulated in TTL^{short}. Our data suggest an association of leukemia cell cycle progression and engraftment in the NOD/SCID/huALL xenograft model.

Beta-CATENIN is involved in WNT-signaling and cell adhesion and is also associated with apoptosis inhibition and cell growth in other haematological malignancies. ANNEXIN I is involved in the regulation of inflammation and reported to be up-regulated in hairy cell leukemia. Both proteins show high levels in TTL^{short} leukemia samples.

PKC alpha has previously been reported to induce apoptosis in an ALL-cell line [224] and to be up-regulated in non-relapsed pediatric T-ALL patient samples (please see Main Topic results). In line with this we found higher activation of PKC alpha in the TTL^{long} subgroup, indicating an association with good prognosis.

Ongoing studies are needed to further evaluate the role of those molecules as new prognostic markers and therapeutic targets

SECONDARY TOPIC III:

"PHOSPHOPROTEOMICS AND PERSONALIZED THERAPY: PHARMACOTHERAPY PATIENT'S RESPONSE PREDICTION USING REVERSE PHASE PROTEIN ARRAYS"

In collaboration with Prof. Sandra Marmiroli, University of Modena and Reggio Emilia

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5.1 INTRODUCTION

Mapping of deregulated kinases and protein signalling networks within tumors can provide a means to stratify patients with shared biological characteristics to the most optimal treatment and identify new drug targets. In particular, the PI3K/AKT/mTOR signalling pathways are frequently activated in blast cells from patients with Acute Myelogenous Leukemia (AML). AML is a neoplastic disorder characterized by the accumulation of genetically altered myelogenous cells displaying deregulated intracellular signalling pathways and aggressive clinical behavior with poor prognosis. The majority of AML patients achieve complete remission thanks to aggressive therapies but many of them still experience relapse. A great interest is therefore focused on the identification of new therapeutic strategies to improve the treatment of this disease.

5.2 AIM OF THE STUDY – SECONDARY TOPIC III -

The objective of the study was to characterized signal transduction pathways in AML adult patients by means of RPPA and to predict the sensitivity of each

patient to PI3K/Akt/mTor inhibitors, to avoid unnecessary and toxic ineffective treatment of non-responsive patients

5.3 MATERIALS AND METHODS

Using Reverse Phase Protein Arrays (RPPA), we analyzed the phosphorylated epitopes of signalling proteins of 50 peripheral blood and bone marrow specimens with newly diagnosed AML. Patients were classified according to blast content, FAB classification and cytogenetic analysis. Samples were enriched for leukemic cells by performing Ficoll separation to yield a mononuclear fraction with >60% blast cells. Fresh blast cells were grown for 16 h untreated or treated with the mTor inhibitors Torin1 and Sirolimus (phase II), and the PI3K/Akt inhibitors Perifosine (phase II), AktInhVIII (phase I), Triciribine (phase I), all at high nanomolar-low micromolar dose, either alone or in combination. Then cells were centrifuged and proteins extracted with a buffer suitable for both RPPA and western blotting analysis. Identification of activated proteins was obtained through Wilcoxon tests or two-sample Welch t-tests implemented in multtest package. *P values* were corrected for multiplicity using Benjamini-Hochberg method to control false discovery rate or with the Bonferroni method to control family wise error rate. Heatmaps were generated to highlight the relationships between clustering and protein expression level.

5.4 RESULTS

Remarkably, by unsupervised hierarchical clustering a strong phosphorylation/activity of most of members of the PI3K/Akt/mTOR pathway was observed in 70% of samples from AML patients. This confirms that this pathway might indeed represent a pharmacological target in many patients. "High ph-Akt" patients blast cells were thus treated with the above inhibitors and we have been able to discriminate a PI3K/Akt/mTOR inhibitor-responsive group of patients and a PI3K/Akt/mTOR inhibitor non-responsive group. In addition, our data indicate that the Akt pathway is hyper-activated in M4, M5 patients, compared to M1, M2 patients, and that a strong activation of most upstream and

downstream Akt effectors correlates with an over-expression of the c-kit receptor (CD117). The data obtained with RPPA were confirmed by both western blotting and flow cytometry analysis. We believe these data are important because they have the potential to define a profile for the personalized administration of targeted drugs.

5.5 CONCLUSIONS

Reverse Phase Protein Arrays allowed to study signal transduction pathways in a wide number of samples, obtaining new information about protein expression and activation in AML patients. We confirmed that PI3K/Akt/mTOR pathway could be considered an important therapeutic target in AML therapy. Moreover we proposed a useful model that allows to predict response to PI3K/Akt/mTOR pathway inhibitors.

SECONDARY TOPIC IV:

"PHOSPHOPROTEOMIC PROFILE IN PEDIATRIC B-LINEAGE LYMPHOBLASTIC LEUKEMIA: IDENTIFICATION OF NEW PROGNOSTIC MARKERS AND THERAPEUTIC TARGETS"

In collaboration with Dr. Tumino Manuela and Dr. Lo Nigro Luca, University of Catania

Tumino Manuela, Accordi Benedetta, Sciro Manuela, ***Milani Gloria****, Tognazzo Federica, Giordan Marco, te Kronnie Geertruy, Basso Giuseppe, Lo Nigro Luca*
"Expression of Annexin 2 In Pediatric B-Acute Lymphoblastic Leukemia: A Marker Of Aggressiveness And Potential Therapeutic Target"

51st Annual Meeting of the American Society of Hematology (New Orleans, LA - Dec. 5th-8th 2009) (Poster)

6.1 INTRODUCTION

Acute Lymphoblastic Leukemia (ALL) is the most frequent cancer in childhood. Although current chemotherapy protocols reach an event free survival (EFS) greater than 70%, the remaining cases suffer of relapse and ALL is still the first cause of death in children with cancer. The majority of ALLs are of B-cell lineage origin in both children (>80%) and adults (>75%). In B cell precursor ALL (BCP-ALL) blast cells are regarded as malignant counterparts of normal B-cell precursors. Several efforts have been made to better understand the underlying mechanism of causing ALL, but current chemotherapy protocols have reached a plateau of effectiveness. The detection of minimal residual disease (MRD) during treatment leads us to stratify children with ALL in standard (SR), intermediate (MR) and high (HR) risk, respectively. In the latter group patients

with a prednisone poor response, with t(9;22) and t(4;11) are included (HR by default). Despite of this improved definition of therapeutic response, the majority of patients who will relapse are initially stratified as MR risk. For this reason new biomarkers and molecular targets are needed. The objective of functional proteome analyses of ALL is to bridge clinical applications with new biomarker discovery that will be useful for determining prognosis and response to therapy already at diagnosis and to identify potential new personalized treatments.

6.2 AIM OF THE STUDY – SECONDARY TOPIC IV -

The aim of this study was to analyze the phosphoproteomic profile in pediatric BCL-ALL patients by RPPA approach, in order to point out differently expressed and activated proteins and identify new candidate biomarkers and therapeutically targets.

6.3 MATERIALS AND METHODS

81 diagnostic samples (peripheral blood and/or bone marrow) with BCP-ALL were analyzed, diagnosed and treated at Padova or Catania centers and enrolled in AIEOP-LLA 2000 Protocol. Multiplexed phosphoproteomic analysis (RPPA) was used to interrogate the expression of 81 phosphorylated or native protein endpoints. We evaluated Pro- and Anti-Apoptotic, Protein Kinase, and Differentiation cell signaling pathways. For statistical purposes, we considered the following characteristics: age at diagnosis, gender, immunophenotype, karyotype, white blood cell count and percentage of blast at diagnosis, response to prednisone, level of MRD at day 33 (TP1) and day 78 (TP2) during Induction phase, outcome. We distributed patients in 4 groups, based on risk (21 SR, 31 MR, 10 HR) and occurrence of relapse (19). We also studied patients from both diagnosis and relapse only in those samples with a blast rate greater than 50% at relapse (10 cases out of 19). All findings obtained with RPPA were confirmed by Western Blot analyses. We applied the Wilcoxon Test and the Wilcoxon Paird Test using the R-project software. *P values* were corrected to control family wise

error rate by means of Holm method. An unsupervised analysis and a multivariate analysis were also performed.

6.4 RESULTS

Preliminary analyses comparing patients subgroups for gender, immunophenotype (Common vs others), karyotype (t(12;21) positive vs negative), prednisone response (Prednisone Good Responders vs Prednisone Poor Responders), MRD stratification (SR vs MR, MR vs HR, SR vs HR, MR vs MR relapsed) and matched diagnoses vs relapses, did not highlight any statistically significant result. A statistically significant higher expression of FosB and Annexin II resulted in the group of children with WBC >50000/mmc (p value <0,05). We also found that a specific pattern of expression (high PTEN (S380), PARP cleaved, Caspase 7 cleaved, PDK (S241), PKAc (T197), p90RSK (S380), MEK ½ (S217-221), IKBα (S32), GRB2 and low Beta-Catenine) characterize a subgroup of children (n°9) with a better survival when compared with other patients (*p value* <0,05).

Moreover, we analysed the 10 proteins with the highest variance between diagnosis samples. We performed a multivariate analysis to find a relation between protein expression/activation and clinical and molecular variables such as sex, age, immunophenotype, DNA index, chromosomal translocations, Prednisone response, WBC count, Minimal Residual Disease, relapse incidence and survival. More interesting results concern the positive relation between relapse occurrence and C-JUN (p=0.0002), ABL T735 (p=0.0117) and CREB S133 (p=0.0072) expression. CREB S133 resulted positive related also to age at diagnosis higher than 10years (p=0.026) and GSK3 a/b S21/9 resulted positive related to t(9;22) (p=0.027) and to Prednisone Poor Response (p=0.0338).

6.5 CONCLUSIONS

RPPA data will be deeper analyzed, but these preliminary data already show that RPPA, associated by a confirmatory Western blot analysis, is a reliable strategy for identification of biomarkers and/or molecules for targeted therapy.

Application of this methodology on specific subgroup of cases with ALL will lead us to identify new therapeutic strategies.

SECONDARY TOPIC V:

"SCREENING OF PP2A ISOFORMS GENE EXPRESSION IN T-ALL REVEALED PP2AR5D UPREGULATION AT DIAGNOSIS IN RELAPSED PATIENTS"

7.1 INTRODUCTION

PP2A is an ubiquitous and conserved serine/threonine phosphatase, with broad substrate specificity and diverse cellular functions. Its role is to reverse the action of kinases in most major signalling cascades. It could function as negative regulator of cell growth and carcinogenesis but it has also a positive role in cell proliferation control [225]. PP2A can either promote or inhibit apoptosis, depending on the cell type and the presence or absence of particular apoptosis-inducing stimuli [226][227]. PP2A has been described as a potential therapeutic target in Chronic Myeloid Leukemia, Philadelphia chromosome-positive Leukemia, Acute Lymphoblastic Leukemia and B-cell Chronic Lymphocytic Leukemia. PP2A inactivation is a recurrent event also in Acute Myeloid Leukemia (AML) and downregulation of some PP2A subunits might contribute to PP2A inhibition in AML [228]. The decrease of PP2A B55α expression in AML is at least partially responsible for increased AKT signalling and it is thus considered as an adverse prognostic factor in AML [77].

PP2A is a heterotrimeric phospatase and it consists of a dimeric core enzyme, composed of the structural subunit A, the catalytic subunit C and the regulatory subunit B. When the PP2A catalytic C subunit associates with the A and B subunits several species of holoenzymes are produced with distinct functions and characteristics. The A subunit is the scaffold required for the formation of the

heterotrimeric complex. After A subunit binding, it alters the enzymatic activity of the catalytic subunit C, even in the absence of B subunit. Regulatory B subunits play key roles in controlling the localization and the specific activity of different holoenzymes, but the precise role of the individual PP2A B regulatory subunits remains undisclosed. There are 4 classes of regulatory subunits: B (PR55), B′ (B56 or PR61), B″ (PR72), and B‴ (PR93/PR110), with at least 16 members in these subfamilies. (Fig. 7.1) Moreover, accessory proteins and post-translational modifications (such as methylation) control PP2A subunit associations and activities [229][230].

7.2 AIM OF THE STUDY – SECONDARY TOPIC V -

A screening of mRNA expression of different PP2A isoforms was performed in T-ALL patients at diagnosis by means of RQ-PCR in order to identify new prognostic biomarkers or therapeutic targets clinically relevant in this disease.

7.3 MATERIALS AND METHODS

7.3.1 RNA extraction and cDNA synthesis

T-ALL patients total RNA was isolated using TRIZOL reagent, following manufacturer's instruction (Invitrogen, Paisley, UK). The RNA quality and concentration were assessed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.), respectively.

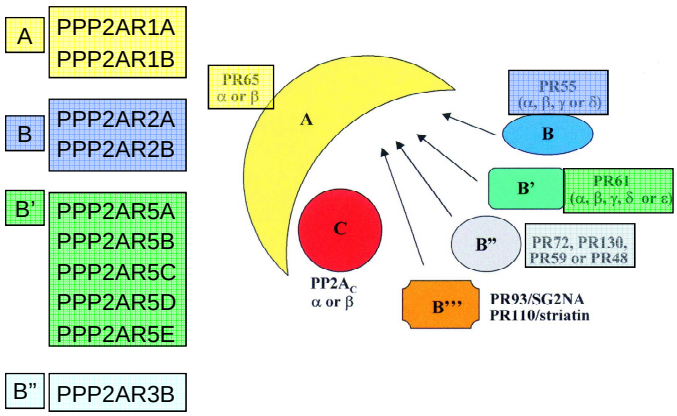

Fig. 7.1: PP2A subunits and isoforms. Studied genes are specified in the left.

cDNA was synthesized using SuperScripII reverse transcriptase (Invitrogen) starting from 1µg of total RNA. RNA was primed at 75°C for 10 minutes and then the reverse transcriptase reaction (RT-PCR) was performed in a total volume of 20µl as following: 25°C for 10 minutes, 42°C for 30 minutes, 99.9°C for 5 minutes.

7.3.2 RQ-PCR analisys

PP2A isoforms expression of different subunits (A, B, B') was quantified in 39 T-ALL patients at diagnosis using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) through Real time Quantitative PCR (RQ-PCR), using the 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The housekeeping gene *GUS B* was chosen as reference. Primers sequences were published by Roberts KG et al. [231] except for *PPP2R2B* and *PPP2R2C* that were designed using Primer Express Software (Applied Biosystems). *PPP2R2B* and *PPP2R2C* primers were designed to amplify sequences that contain introns, so that spliced mRNA-derived signals could be distinguished from any contaminating genomic DNA-derived signals by their length. To exclude contamination of unspecific PCR products, melting curve analysis was always applied to final PCR products after the cycling protocol. A serial dilution curve of a T-ALL cell line was always amplified in parallel to be used as internal control. cDNA from normal T cells was used as calibrator. *PP2A* different isoforms expression levels were determinated using the $2^{-\Delta\Delta Ct}$ method.

In Table 7.1 different PP2A isoforms of different subunits studied and primers sequences used are listed.

7.4 RESULTS

In the first place, the mRNA expression of different isoforms of PP2A subunits, described in Table 7.1, was studied by RQ-PCR in 24 T-ALL pediatric patients at diagnosis. PP2A isoforms expressions were compared between T-ALL patients who relapsed and who not relapsed. Among different PP2A isoforms, PPP2AR5A, PPP2AR5D and PPP2AR5E resulted differently expressed between

patients who encountered relapse (N=12) and patients who did not encounter relapse (Wilcoxon test, $p<0.05$) (see Fig. 7.2).

Table 7.1: RQ-PCR PPP2A and GUS Primers Sequences [231]**.**

GENE STUDIED	Primer Sequence 5'→ 3'
PPP2R1A	F - GCTCTTCTGCATCAATGTGCT R - ATGCGCAGAACCGTGGGTA
PPP2R1B	F - ACTTTATTCTGCATTAATGCACT R - TGGGCAGCATTTGCTTAGTA
PPP2R2A	F - CTGTGGAAACATACCAGGTG R – AACACTGTCAGATCCATTCC
PPP2R2B	F – ATCCTGCCACCATCACAAC R - GCTTCTCATAAACGGTTGCG
PPP2R2C	F – GAGGGGAAACTTAAGGACCTG R - ACTCGGGAGCCTCCTAG
PPP2R2D	F - AACGGTTCGGATAGCGCCA R - GCGTGTCTCTATCAAACATC
PPP2R5A	F - TTGTTTCATGCTCAGCTAGC R - GGCTCTGTTAGTGTTGTATC
PPP2R5B	F - ACTCTGACAGAGCACGTGAT R - GAAACATCACCTCCTTCTGG
PPP2R5C	F - TGGCCTCATCTACAGCTTGT R - GGTTGGAAATCTGGAGACTCT
PPP2R5D	F - ACTGAGGCTGTTCAGATGCT R - CGCAGCAGCACTTTCTCCT
PPP2R5E	F - TCAGATCGTCAGCGTGAGA R - CTCTTTAACTCCAGATCCTC
GUSB	F- GAAAATATGTGGTTGGAGAGCTCATT R - CCGAGTGAAGATCCCCTTTTTA

This data was deepened by studying the expression of the three isoforms PPP2AR5A, PPP2AR5D and PPP2AR5E in an enlarged cohort of 39 patients, 19 who encountered relapse and 20 who did not relapse. Among these three isoforms, higher expression of PPP2AR5D in T-ALL patients who encountered relapse at diagnosis was confirmed (Wilcoxon test, $p=0.019$) (see Fig. 7.3). Among the

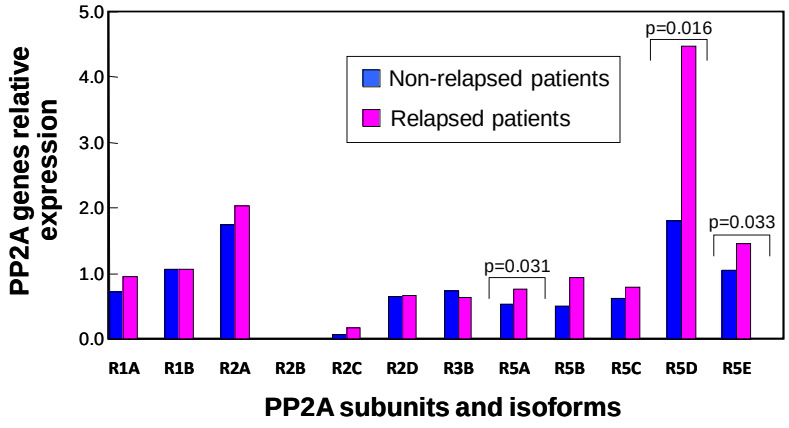

Fig. 7.2: Relative expression of different isoforms of PP2A subunits in T-ALL patients who experience relapse or not.

isoforms of the three analysed subunits, PPP2AR5D resulted to be overexpressed at diagnosis in patients who relapsed.

7.5 CONCLUSIONS

The PPP2AR5D gene showed mRNA overexpression in T-ALL patients who encountered relapse compared to those who did not relapse. This gene encodes the delta isoform of the regulatory subunit B56 subfamily (PP2A B' δ protein). The expression of this isoform of PP2A is thus associated to poor prognosis in pediatric T-ALL.

It is known that B regulatory subunits might modulate substrate selectivity and catalytic activity of PP2A and also might regulate the localization of the catalytic enzyme to a particular subcellular compartment. Little information about specific role of PP2A regulatory B' δ subunit is available. More studies on PP2A B' δ protein functional role are needed to understand its involvement in PP2A activity. Moreover, further analyses on PPP2AR5D higher expression of T-ALL patients who relapsed might elucidate the downstream effects of its upregulation.

PPP2AR5D differential expression, validated in a larger clinical set of T-ALL pediatric patients, could highlight PPP2AR5D as a new useful prognostic biomarker detectable at diagnosis. Moreover, once its functional role in leukemic process is clarified, it could also represent a novel drug target for PP2A inhibitors in T-ALL.

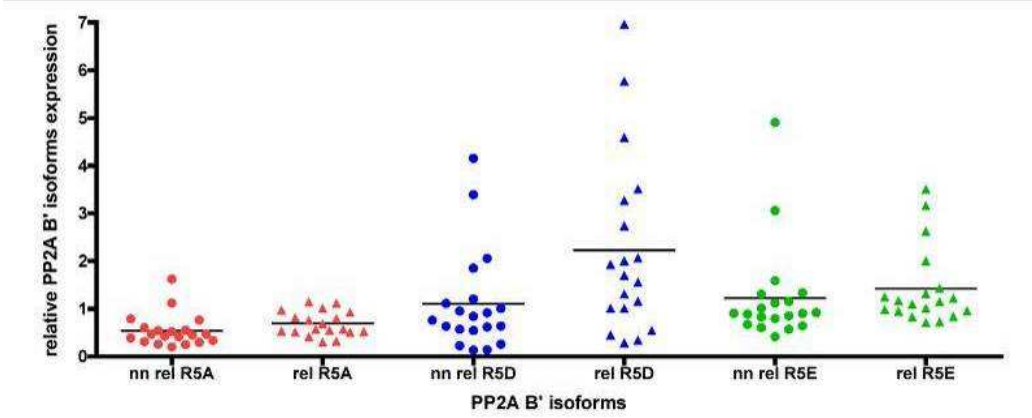

Fig. 7.3: Relative expression of PP2A B' isoforms (i.e. PPP2AR5A, PPP2AR5D, and PPP2AR5E). Cohort of 39 patients, 19 who encountered relapse and 20 who did not relapse.

CONCLUSIONS

During my PhD course I applied the Reverse Phase Protein Arrays (RPPA) technique to the study of patients affected by Acute Leukemia. In particular, in the "Main Topic" of my thesis new data arisen from a phosphoproteomic study of T-ALL pediatric patients at diagnosis are described. In "Secondary Topics" relevant results from BCP-ALL phosphoproteomic profiling has been reported, as well as collaborative national and international research projects that shows other RPPA applications.

The principal aim of this research was the discovery of new candidate biomarkers and therapeutic targets involved in the leukemic process through the study of the phosphoproteomic profiles of ALL pediatric patients (T-ALL and BCP-ALL), investigating the aberrant activation or expression of proteins involved in altered signal transductions pathways by means of the RPPA technique.

Acute Lymphoblastic Leukemia (ALL) is an aggressive malignancy of lymphopoietic cells characterized by a clonal proliferation of blast cells originated from lymphoid precursors arrested at early stages of differentiation. ALL is the most common pediatric cancer, accounting for a quarter of all malignancies among children aged less than 15 years. Nowadays the introduction of risk-directed treatment and intensified cure protocols has improved the outcome of ALL pediatric patients. Indeed, current therapies achieve five-year event-free survival rate of about 80% in children receiving protocol-based therapy in the developed countries. Advances in genomics and molecular analysis have improved the ability to select patients that are likely to respond to a particular therapeutic agent, but substantial numbers of patients still experience relapse and have poor outcomes, failing to ever achieve a complete remission. In order to

develop new additional therapeutic supports and to assemble specific and personalized therapies for ALL pediatric patients, improving drug response and reducing drug toxicity, it is extremely important to identify novel therapeutic targets and new prognostic biomarkers on leukemic cells.

To pursue this aim, I applied RPPA approach searching for new information regarding leukemic cells biology through the study of protein networks activation state. RPPA can measure the activation levels/phosphorylation of large numbers of signalling proteins at once from small clinical samples in a very reproducible, precise, sensitive and high-throughput manner.

RPPA proved to be a powerful tool for profiling and comparing protein pathways to identify novel diagnostic and prognostic biomarkers for analyzed diseases. The identification of critical nodes or interactions within the network is a potential starting point for drug development and/or design of individual therapy regimens. We mainly applied RPPA to translational studies, through retrospectively analysis of patients specimens, but this technique presents a variety of different application as pharmaceutical discovery and clinical application.

In RPPA, a protein microarray immobilizes the proteins to be analyzed on a nitrocellulose-coated glass slide. Each group of spots (undiluted to 1:8 dilution curves in duplicate) represents an individual test sample, allowing an array to be comprised of multiple, different samples. Each RPPA slide is probed with a single detection molecule (one slide = one antibody) and a single analyte is concurrently measured across multiple samples. This format allows multiple samples to be analyzed under the same experimental conditions for any given analyte. This very much facilitates comparisons between samples, since experimental variability is eliminated. The advantage of this method is also the possibility to analyze a wide number of specimens using low sample volumes thanks to a signal amplification technology. These benefits are perfectly matched for molecular profiling of clinical patients samples because frequently only a small amount of patient material is available for molecular analyses. The biggest limitation of RPPA is that its success is highly dependent on the quality of the antibody set used: all the

antibodies employed for the staining have to be extensively validated for single band specificity by Western Blot, to ensure its reactivity and its high specificity.

In the study of pediatric T-ALL reported in the “Main Topic”, we identified PKCα as a new prognostic marker of relapse by analysis of samples at diagnosis. This promising protein kinase has been identified through a retrospective RPPA screening of fifty-three different proteins/phophoproteins in 98 T-ALL specimens. PKCα resulted to be downregulated in the group of patients with a higher incidence of relapse. We showed that PKCα activation is a consequence of a low *PKCα* gene expression . Moreover, we induced PKCα inhibition in T-ALL cell lines using a PKCα commercial inhibitor and an increase in proliferation rate was detected. RPPA thus proved to be a useful approach to discover new molecules aberrantly expressed in leukemia pathways. The identification of players involved in malignant mechanisms allows to focus on novel prognostic factors: PKCα resulted to be a promising candidate marker of risk of relapse in pediatric T-ALL. A study of PKCα expression in a larger T-ALL cohort patients will allow to confirm this relevant data and to propose PKCα as a new prognostic marker for T-ALL stratification.

In the pediatric B-cell precursor (BCP)-ALL study we used RPPA to map in 118 pediatric BCP-ALL patients the pathway activation status of 92 different phosphorylated and total proteins of key signalling “hubs” known to be involved in human tumorigenesis and metastasis. Correlation of signalling activation with clinical response and known genetic information (e.g. gene rearrangements), enabled us to identify new protein pathway biomarkers that, when validated in larger clinical sets, could be used for patient stratification and targeted therapy trials. In detail, we observed an increased activation/expression of several pathways involved in cell proliferation in poor clinical prognosis patients. First, MLL-rearranged tumours revealed BCL-2 hyperphosphorylation through AMPK activation, indicating that AMPK could be able to inhibit apoptosis in MLL-rearranged patients and thus could be considered as a new potential therapeutic target. Second, in patients with poor clinical response to prednisone we observed

the up-modulation of LCK activity with respect to patients with good response. This tyrosine-kinase can be down-modulated with clinically used inhibitors, thus modulating LCK activity could be considered for further studies as a new additional therapy for prednisone-resistant patients. Further we also found an association between high levels of CYCLIN E and relapse incidence. Moreover, CYCLIN E is more expressed in early relapsed patients, who usually show an unfavourable prognosis. We also decided to explore in depth the functional significance of AMPK activation in MLL-rearranged patients. We showed that the AMPK pathway contributes directly to the survival of MLL-rearranged BCP-ALL cells and AMPK can be considered a new druggable target in MLL-rearranged leukemias. To this end, AMPK inhibitors represent a promising therapeutic strategy for these poor prognosis patients.

RPPA reliability in detection of novel targets and markers has been verified also in collaborative studies with national and international research projects, regarding also adult diseases such as Acute Myeloid Leukemia.

In my Ph.D. course I proved that the application of Reverse Phase Protein Arrays is a reliable, sensitive and high-throughput method useful to identify new aberrant molecular players involved in haematological malignancies, in order to discover novel prognostic markers and therapeutic targets to propose for the development of more effective patient-tailored treatments.

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