**Personalizing pediatric acute lymphoblastic leukemia using dynamic BH3 profiling**

**Abstract:**

The most common pediatric cancer is acute lymphoblastic leukemia (ALL). Relapsed patients present few therapeutic options, poor prognosis and resistance to apoptosis. We aim to use a novel functional assay called dynamic BH3 profiling or DBP, that measures early apoptotic events and can accurately predict in less than 24 hours which treatment(s) will be most effective to eliminate a specific cancer. DBP is at present being evaluated in multiple types of cancer and has already been successfully applied in ALL patient-derived xenograft models and patient samples. Our main aim is to use DBP to find the optimal therapies to personalize pediatric ALL patients' treatment, working with pediatric oncologists from the SEHOP (including the relapsed acute lymphoblastic leukemia network ReALLNet).

**Background**

The most common pediatric cancer is acute lymphoblastic leukemia (ALL), affecting 3 out of 4 pediatric leukemia patients. It is characterized by varied genetic alterations that may affect receptors and transcription factors, leading to malignant transformation. Among them we can find Philadelphia chromosome positive (Ph+) B-ALL, JAK2 fusions, CRLF2 increased expression, ETV6-RUNX1 fusion, MLL (KMT2A) rearrangements… Despite this heterogeneity, actual chemotherapeutic regimes used to treat ALL in the clinic reach outstanding results and cure rates of approximately 90% (Adamson, CA Cancer J Clin, 2015). But ~10% of pediatric ALL patients relapse and succumb to the disease pointing to the unmet need for new treatments. Because of this, pediatric cancer is still the main cause of death by disease in children under the age of 15, due to the very low frequency of somatic mutations, thus reducing the number of existing biomarkers and new targeted therapies (Grobner et al., Nature, 2018). Furthermore, pediatric cancer treatment often leads to secondary effects, often causing IQ reduction, cardiotoxicity and later health complications in life (Sarosiek et al., Cancer Cell, 2017). One of the hallmarks of relapsed ALL patients is resistance to apoptosis. In fact, all genetic rearrangements described above regulate BCL-2 family proteins, mostly leading to an increase in antiapoptotic proteins' availability. For example, the BCR-ABL fusion protein found in Ph+ B-ALL upregulates BCL-2; JAK2 signaling increases the expression of BCL-2, BCl-xL and MCL-1; ETV6-RUNX1 promotes BCL-xL expression, etc. (Seyfried et al., Cell Death Dis. 2019; Brown et al., J Biol Chem, 2017; Del Gaizo Moore et al., Blood, 2008). In this regard, understanding how ALL cells adapt to therapy in the clinic and acquire an antiapoptotic phenotype can be useful to design new therapeutic strategies to better treat relapsed/refractory patients.

In this project we will use dynamic BH3 profiling or DBP, a novel functional assay that has the capacity to accurately predict in less than 24 hours which treatment(s) will be most effective directly on patient biopsies (Montero et al., Cell, 2015). When a cancer cell is effectively treated, early changes in the BCL-2 family of proteins ('priming') can be rapidly detected preceding the activation of apoptosis and the cancer cell's commitment to die. By using titrated doses of synthetic BH3 peptides, like BIM BH3, that acts as a catalyst to induce apoptosis, we can anticipate therapies' effectiveness. In other words, DBP measures how much a treatment primes cancer cells for apoptosis (Δ% priming), which is predictive of their cell fate, allowing a rapid analysis of many samples and treatments in a high-throughput manner. It

has been successfully tested in vitro, in murine models and on patient samples (Montero et al., Cell, 2015; Montero et al., Cancer Discov, 2017). We currently use a flow cytometry-based DBP assay (Montero and Letai, CDDiff, 2018; Ryan et al., Biol Chem, 2016), where cells are stained with antibodies against tumor molecular markers, allowing us to select for specific cellular subpopulations in patient samples. This technology represents an enormous technical advantage, because it avoids sample deterioration due to the short ex vivo culture and allows to directly test therapies on patient-isolated cancer cells with an excellent predictive capacity (assessed by ROC curve analyses). Furthermore, DBP has been used successfully in ALL patient-derived xenograft models to determine response to JAK2 and MDM2 inhibitors (Townsend et al., Cancer Cell, 2016; Wu et al., Cancer Cell, 2015), thus justifying its further uses to find new treatments for ALL. This technology is clearly superior to other existing biomarkers and is now being evaluated as a clinical companion diagnostic for multiple types of cancer; having the potential to be used to truly personalize ALL treatment. In fact, my team is already successfully using this strategy for pediatric tumors (Alcon et al., Cell Death Dis. 2020), including ALL (Manzano-Munoz et al., Front Cell Dev Biol., 2021). We are particularly exploring the potential therapeutic sue of BH3 mimetics such as venetoclax that hold great promise for pediatric ALL treatment (Seyfried et al., Cell Death Dis. 2019; Brown et al., J Biol Chem, 2017; Del Gaizo Moore et al., Blood, 2008; Manzano-Munoz et al., Front Cell Dev Biol., 2021). In this project, we intend to collaborate with pediatric oncologists to identify and test new treatments directly on pediatric ALL patient samples (focusing on relapsed/refractory) to improve personalized treatment while reducing the devastating secondary effects often observed in the clinic (Sarosiek et al., Cancer Cell 2017).

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**Hypothesis And Objectives**

We propose to use the novel functional assay dynamic BH3 profiling to study ALL biology, and study the relationship between active signaling pathways and apoptosis to overcome resistance to therapy. We will use DBP to evaluate new therapeutic strategies for the first time directly on primary relapsed pediatric ALL patient samples to help personalize treatment and improve survival in the clinic. We will work in collaboration with Dr. Manuel Ramírez Orellana (Head of Unidad De Terapias Avanzadas, Servicio de Oncohematología, Hospital Universitario Niño Jesús) and SEHOP oncologists - including the relapsed acute lymphoblastic leukemia network (ReALLNet) coordinated by Dr. Pablo Velasco (Vall d'Hebron) and Dr. José Luís Fuster (Hospital Universitario Virgen de la Arrixaca) with whom we already started to collaborate performing DBP. To our knowledge, this project is the most comprehensive effort to use a state-of-the-art functional assay to guide precision medicine and find new therapies, to improve relapsed pediatric ALL patients' treatment that present very poor survival rates.

**AIM 1. Identification of apoptosis regulators and new therapies in ALL cell lines.**

Identify which drugs induce apoptosis: We will select representative treatments of different classes of anticancer agents and evaluate apoptotic engagement. We will first test DBP's predictive capacity in ALL cell lines and identify promising treatments.

We will use multiple pediatric and young adult ALL cell lines (like NALM-6, SEM, Jurkat and others), including B-cell and T-cell ALL, expose them to different treatments and perform flow cytometry-based DBP. We anticipate using 5-10 ALL cell lines. Based on the available information for each cell line, we will select representative treatments for each class of anticancer agents and perform DBP to identify which ones induce the greatest increase in priming and engage apoptosis. We will focus in the study of key membrane receptors (different tyrosine kinases, BCR-ABL, FLT3…), relevant signaling pathways (like MAPK, PI3K/mTOR, WNT, JAK proteins...), cell cycle, DNA repair and others. In this regard, we will prioritize representative families of compounds: conventional chemotherapy (cytarabine, daunorubicin, L-asparaginase, vincristine, methotrexate, prednisone, and others), targeted therapies (including imatinib, dasatinib, ruxolitinib, sunitinib, trametinib, FLT3 inhibitors, WNT inhibitors, etc.), BH3 mimetics (venetoclax, A-133, DT2216 or S63845) and others, prioritizing those that are either approved for the clinic or in clinical trials. Briefly, we will emphasize not only in drugs currently used at the hospital, but we will try to identify novel cytotoxic compounds exploiting ALL oncogenic addiction. We anticipate working with an initial panel of approximately 15-30 compounds or combinations. If other drugs of interest are identified, like new classes of active compounds or novel agents against specific targets, we

will include them in our panel. We will perform DBP, determine if apoptosis will be engaged and validate the measurements with in vitro cytotoxicity cell death analyses (Annexin/PI staining). These first experiments will be performed as a proof of principle, preceding patient samples' analyses in Aim 2. We will use these tests to compare Δ%priming with %cell death and run a ROC curve analysis to determine how good of a binary predictor DBP is for ALL. In fact, we already have promising preliminary results pointing in this direction with the NALM-6 and SEM cell lines.

**AIM 2. Personalizing ALL treatment in the clinic**

Analyze pediatric ALL patient samples. In collaboration with ALL expert oncologists at SEHOP and ReALLNet we will test the standard-of care and selected therapies from aim 1 to perform drug response profiling on different therapies using DBP analyses. We will use the experience acquired to analyze pediatric ALL primary samples and help oncologists improve treatment in the clinic, especially for relapsed patients, in an unprecedented precision medicine effort integrated in ReALLNet.

Approximately 300 new cases are diagnosed each year in Spain, a quarter of all pediatric malignant tumors, according to the Spanish registry of childhood tumors (RETI-SEHOP). With current treatment regimens, around 15% of pediatric patients with acute lymphoblastic leukemia relapse (ped R/R ALL) and, after relapse, the overall survival is reduced to less than 50%. We will work with the ReALLNet and SEHOP (see letters of support) and they agreed to share with us Ficoll-purified bone marrow aspirates or peripheral blood from relapsed pediatric ALL patient samples. The ReALLNet has a board of clinical and biological experts to fully evaluate relapsed ALL cases presented by treating physicians - this group is already constituted and functional (ReALLBoard). Within the ReALLBoard we are currently developing the resources to create a national biobank for ped R/R ALL and the resources to facilitate the oncogenomic and functional diagnosis to all the centers that need it, as well as to connect these 2 networks within the resources created to respond to cases and register patients. In this context, the ReALLNet Board has agreed that Dr. Montero's team will perform functional drug response profiling study within the project, integrating the results of its analysis to the oncogenomic tests and thereby guiding therapeutic recommendations in a more profound and effective way.

Dr. Montero's laboratory will work both with freshly obtained and viably frozen samples. We anticipate that the study will recruit 30-40 ped R/R ALL patients per year. We will work in collaboration with Prof. Pablo Menéndez (from Josep Carreras Leukemia Research Institute) laboratory, as they will also perform ex vivo drug response profiling analyses. R/R ALL patients present few therapeutic options but could benefit from compassionate drug use and we will use DBP in order to find better treatments for the most complicated cases. Samples provided will be collected in the framework of another research project within their clinical institution, through informed consent. The conditions and purpose of this donation will be clearly detailed to the patient before extraction by qualified personnel of the hospital, including the fact that donated samples will be exclusively used for research and that his/her personal and clinical data will be protected according to the national and European law for data protection. We estimate that ~20% of consented patients might not provide useful information. ALL patients included in this proposal will provide significant results to fulfill the objective of Aim 2. Kappa and other concordance index will be used to correlate treatment response prediction in patient samples and matched PDXs. Long-rank test and Kaplan-Meier will evaluate the impact of cell functional assay and their shift on DFS and OS. As per response prediction, McNemar paired test will determine performance of pre-treatment and post-treatment profiling after variables categorization by oncologists.

**Project Resources and Costs**

The group of Dr. Montero will be located at the Department of Biomedical Sciences at the Faculty of Medicine and Health Sciences at the University of Barcelona (UB) and collaborating with the Institute for Bioengineering of Catalonia (IBEC) and the Josep Carreras Leukemia Research Institute. The UB is one of the top research universities in Spain. Dr. Montero´s laboratory will have access to all the services and technologies offered by the Scientific and Technological Centers of the University of Barcelona (CCITUB) which include Advanced Microscopy, cytometry, proteomics and animal facility, among others. The laboratory of Cell Biology (Dept of Biomedicine) is in the Campus Clínic, and this is remarkable for the development of this proposal. Indeed, the Campus Clinic hosts several institutions: the Faculty of Medicine and Health Sciences (UB), the Hospital Clínic, the August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Josep Carreras Leukemia Research Institute (Campus Clínic) and the Spanish National Research Council (CSIC). This will facilitate the collaboration of Dr. Montero's laboratory with clinical researchers, and also the access to the IDIBAPS Biobank and the scientific platforms of IDIBAPS, being the Cytometry and Cell Sorting facility, of special interest for this research. The team involved in this project will be composed by Dr. Joan Montero (PI), Dr. Clara Alcon (Senior technician) and Albert Manzano (last year PhD student), that already generated preliminary results inspiring this proposal.

Funds are requested for cells lines, cell culture medium, growth factors, serum, trypsin, plasticware, glassware, fluorescently labelled antibodies, synthetic peptides, enzymes for sample digestion, fluorescent dyes, reagents/consumables needed for in vivo experiments and other chemical reagents. We also request funds for the use of scientific services such as flow cytometry facilities (CCITUB/IDIBAPS),