Proteomic tools and new insights for the study of B-cell precursor acute lymphoblastic leukemia

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1. Introduction

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a hematological malignancy characterized by uncontrolled clonal expansion of B-lymphoid progenitors in the bone marrow (BM) and extramedullary sites. In childhood, BCP-ALL represents up to 80% of the total diagnosed cases of acute lymphoblastic leukaemias (ALL) [1]. The global incidence of BCP-ALL is 1–5 cases per 100,000 people [2], but it differs between countries. For example, the incidence of BCP-ALL in The United States is 11 cases per 1,000,000 people [3] corresponding to 20% for young population with ages between 1 and 20 years (i.e. 3000 new cases per year) [4], but only 12% of total cases of cancer for ages between 1 and 20 years (i.e. 3000 new cases per year) [4], but only 12% of total cases of cancer for ages between 1 and 20 years (i.e. 3000 new cases per year) [4]. In upper middle and middle-income countries like Mexico, the incidence of ALL is 43.2–44.9 cases per 1,000,000 people [6] of which, BCP-ALL represents between 73.2% and 76.1% of total diagnosed cases with a peak of incidence between 2 and 6 years old [6,7].

The etiology of BCP-ALL remains unknown, but the important role that genetic factors have in the development of leukemia has been widely documented [8]. Genetic abnormalities along with immunophenotyping of leukemic cells as well as blood cells counting parameters are commonly used for diagnosis and for the establishment of the prognosis of disease. However, because genetic abnormalities and alterations are different in many BCP-ALL cases [9] is necessary to count with appropriate tools for the study of the etiological basis of the disease and to conduct a risk stratification for a precise targeted therapy for BCP-ALL. Among these tools, omics technologies like proteomics have impacted the study of different diseases [10]. In BCP-ALL, proteomic tools have awakened the scientific community attention because the possibility to evaluate the complete proteome in each stage of the disease and it opens the opportunity to establish their functional participation along of the BCP-ALL progression and its treatment [11]. At the same time, the implementation of appropriate protocols for molecule detection, which consider the origin, the collection and the correct storage of the sample, is a prerequisite before to start a proteomics study. In this review, we will describe the use of proteomic tools in the study of etiological basis and the discovery of molecular biomarkers. Furthermore, in the context of clinical investigation we described the application of proteomic technologies as tools for diagnosis, prognosis, and the search for new biological targets as well as for monitoring of minimal residual disease (MRD) of BCP-ALL.
2. The proteome and the current tools for its evaluation

Proteomics study the structure and function of the proteome [12], namely, the complete set of expressed proteins. The proteome represents the actual functional state of an organism, whose protein expression is the result of stimuli of different factors both external and internal. Therefore the expressed proteins, are regulated by the metabolism of an individual and at molecular level the RNA transcription, alternative splicing, and/or post-translational modifications are some of the processes that modulate that expression. In comparison with the relatively steady state of the genome [13], protein expression at the time that represents the dynamic status of the organism offers an unparalleled opportunity to measure and quantify protein changes as a direct indicator of an ongoing disease process [14]. Proteomic work in patients with BCP-ALL to date has been carried out mainly with body fluids such as cerebrospinal fluid (CSF) peripheral blood (PB) and bone marrow (BM) [15]. The sample preparation of body fluids is an important and critical pre-requisite to achieve efficient data for proteomics [16]. The sample collection for protein analysis is mainly from BM aspirates or blood samples. The lymphocytes from the samples (BM and PB) can be isolated using Ficoll, while the cells from the CSF can be isolated by centrifugation. All cells from the three detailed kinds of samples must be lysated by using kits for human cells (i.e. RIPA). Protecting proteins from the action of proteases and other enzymes by degradation is a critical consideration for ensuring that analytical results can be trusted to mirror in vivo state of the proteins or proteomes assayed in tissues-and cell-based samples. The time, the reagents used and the storage of the sample during its processing are key points to avoid the degradation of proteins [15]. The success of this proteomic research is mainly attributed to the relative ease of sampling and the availability of advanced molecular technology [17].

Once the sample has been prepared, the evaluation of protein expression may be carried out using a series of high-throughput technologies such as mass spectrometry (MS), protein arrays (e.g. RPPA) alone or in combination with other tools like two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and western blot (WB), whose ultimate goal is to identify and quantify the abundance of proteins under specified conditions. In the context of BCP-ALL if we need to evaluate surface or cytoplasmic protein markers in a living cell, sorting cell tools like flow cytometry (FC) has been proved to be useful. The obtained information can then be used for diagnosis, determination of the cell maturation stage, and the monitoring of MRD during and after BCP-ALL treatment [18]. While the technologies mentioned are useful in the study of human diseases, to potentiate its utility, and to analyze the results obtained by proteomic approaches is necessary the conjunction with bioinformatic tools. Bioinformatics includes a growing number of databases such as NIST/EPA/NIH mass spectral library, NIST tandem (MS/MS) library (https://chemdata.nist.gov/), NIST17 (nistmassspectrlibrary.com), MS – Sadtler AAFS toxicology section: mass spectra of drugs, and/or SWGDRUG library (http://www.bio-rad.com). These databases containing information about the structure of proteins, which facilitates the identification of their functions in the development of diseases before any validation at clinical level (Figure 1). Furthermore, proteomic tools could represent new opportunities to increase the available information about the etiological basis of the disease and to understand how the proteome dynamics and changes can influence the onset of BCP-ALL.

2.1. Flow cytometry as an important tool for immunophenotyping normal and malignant B-cells

BCP-ALL originates from B-cell precursors and its detection, classification and the phenotypic features of maturation arrest are mostly based on surface markers, where FC has become routinely used [19]. Plastic niche of ALL represents a challenge for the correct early diagnosis of BCP-ALL. Together with cytomorphology and cytochemistry, FC immunophenotyping is crucial for the detection of blast cells in suspected samples, including the definition of acute leukemias of ambiguous lineage. Comparison of the immunophenotypic features of blasts cells versus normal hematopoietic precursors and immature cells contributes to the definition of the stage of maturation arrest of the blast population within the B- and T-lymphoid lineages as well as the neutrophilic, monocytic, megakaryocytic or erythroid lineages. FC immunophenotyping has also proven to be of great utility for sensitive detection of low levels of residual blast cells and their distinction from normal regenerating immature cells in the BM of ALL patients during treatment [20–23].

Specific phenotypic characterization of ALL blast cells is usually performed using lineage associated markers such as CD19 for B-cells [24]. Accordingly, in B-lineage ALL the most important markers for differential diagnosis and subclassification are CD19, CD20, CD22, CD24, and CD79a [25]. The combined positive and negative reaction for these and other complementary markers identifies blast cell maturation stage which has proven to be of unequivocal clinical value [26]. Based on classical immunophenotyping, BCP differentiation has been subdivided into distinct stages including pro-B (CD22⁺CD19⁺CD10⁻CD34⁺), pre-B-I (CD19⁺CD10.high⁺CD34⁺cyt.µ⁺), pre-B-II-Large
(CD19+CD10−CD34−cyIgμ+CD20high), pre-B-II-Small (CD19+CD10−CD34−cyIgμ+CD20low) and immature (CD19+CD10+IgK/λ+) stage [27].

The BCP-ALL protocol based in EuroFlow Consortium panels is designed to distinguish normal or regenerative precursor B-cells from BCP-ALL blast cells and for full maturation-based classification of BCP-ALL according to EGIL (European Group for the Immunological Characterization of Leukemias) criteria or WHO (World Health Organization) guidelines [21,28]. In the EuroFlow antibody panel (version 1.10), the markers included for the BCP-ALL evaluation are CD9, CD10, CD13, CD15, CD19, CD20, CD21, CD22, CD24, CD33, CD34, CD38, CD45, CD58, CD65, CD117, CD123, SmIgK, CyISmIgλ, CyIgμ, SmIgM, NG2 and NuTdT. From these, the B-lineage-associated markers included are CD22, CD24, CD10, CD20, CyIg, SmIg. The markers with usefulness for differential diagnosis with other acute leukemias include CD13, CD33, CD117, CD15, CD65 (to exclude AML). Other markers suitable for comparison with and distinction from normal B-cell development patterns are NuTdT, CD10, CD38, CD20 and CD123 [21].

The prognosis of the disease is also important and help to apply better treatments to improve the survival rate of leukemic patients. To determine treatment response and subsequent prognosis in patients treated for BCP-ALL, MRD (by the presence of 0.01% or more ALL cells) measurements should be performed [24]. Given that the immunophenotype of leukemic cells resembles that of BCPs, it is particularly difficult to detect residual leukemic cells during therapy intervals, i.e. when regenerating BCPs are abundantly present. Surface molecules like CD34 and CD38 commonly aimed at the identification of leukemic-stem cells (LSC) have been observed differentially expressed in some cases of BCP-ALL and rendered as promising prognostic markers for this malignancy [29].

For MRD evaluation, the markers suggested are CD10, CD19, CD20, CD34, CD38, CD45, CD73, CD81, CD123 and CD304 [21]. The CD19, CD34, CD45, CD10, CD38, CD20 combination is highly efficient in discrimination of normal/regenerating versus malignant immature B-cells and could represent an MRD-oriented combination [21,30]. With the advent of new technologies, the panel of new markers has been increasing; while some functions of these markers are known, many of these remain to be studied. Markers currently used in the clinical practice are CD10, CD19, CD20, CD22, CD34, CD45, CD123, CyIgμ, SmIgK, CyISmIgλ and NuTdT [26,31] (Table 1).

Notwithstanding the advances in FC BCP-ALL immunophenotyping and of the accumulated information, there are a growing number of investigations focused in to improve the correct distinction and sub-classification of different stages of B-cell maturation and their impact in the prognosis of children and adults with BCP-ALL. On this sense and in order to conduct the correct subclassification of BCP-ALL from normal lymphoid precursor hematogones, the expression of relevant surface markers was analyzed by Sedek et al. [32]. Multiparameter FC analysis of CD10− and CD10+ blast cells showed differential expression of molecules compared to hematogones. However, CD10+ blast cells were characterized for a higher expression of CD45, whereas CD10− blast cells showed increased
expression of intranuclear terminal deoxynucleotidyl transferase (TdT), CD22, CD34 and CD20, but a decreased expression of CD45 [32]. Even more, FC have permitted to discover a significant differential expression between children and adults diagnosed with BCP-ALL. Seegmiller et al., by using FC analyzed 200 samples from BCP-ALL patients which received a chemotherapy regimen identified 31 different cell-surface markers including B-, myeloid, T-lymphoid and NK-cell antigens [33]. However, a decreased expression of HLA-DR along with a null expression of CD10 and overexpression of CD45 were more commonly found in adults than children. These studies not only demonstrated the heterogeneity and differential expression in BCP-ALL but also for different age groups and could partially explain the different outcomes observed after the chemotherapy regime.

As mentioned before, after chemotherapy, remnants of blast cells are one of the most important prognostic factors for BCP-ALL patients [34–36]. While FC has become an important tool for the detection of possible MRD, the modulation of surface antigens during therapy of B-ALL can affect the results [37]. For example, analysis by FC of BM from children with BCP-ALL at 15 and 33 days of treatment showed a downregulation of the surface markers CD10, CD19 and CD34, and increased expression of CD20; however, no changes in the expression of CD38, CD58 and CD45 was reported [38]. To overcome this challenge, Theunissen et al., performed the standardization of different markers to improve the specificity and sensitivity of MRD diagnostic based on FC. They standardized two different sets of antibodies to analyze different BM samples derived from pretreated and relapse patients with BCP-ALL. The use of the antibodies CD19, CD10, CD20, CD34 and CD45 in combination with CD38, CD81 and CD15/NG2, could discriminate normal and leukemic cells in 99% of analyzed samples [39]. Furthermore, FC showed a high degree concordance of 98% in comparison to the specificity of Polymerase Chain Reaction (PCR)-based MRD detection [39,40]. In a similar manner, the feasibility of CD73 (in adults) and Neurophilin-1 (NRP-1)/CD304 (in children) as molecular markers for MRD monitoring in B-ALL, has also been demonstrated [41]. In this way, FC is a powerful tool for detecting molecular changes and it could help in the detection of residual malignant cells after therapy. However, it should be kept in mind that, as long as we have more and better markers, the detection of leukemic cells will be a quicker and easier task, thus strengthening the application of FC-based methodologies. Additional examples of protein markers with usefulness in the study and clinical management of BCP-ALL are described in Tables 2 and 3.

### 2.2. Mass spectrometry in the establishment of the molecular basis of leukemogenesis and in discovery of molecular markers for BCP-ALL diagnosis, prognosis and patient response to treatment

MS is a high-throughput technology has been used for quantitation and identification of unknown molecules and for the investigation of different pathologies such as rheumatoid arthritis, diabetes, heart diseases and different cancers, including leukemia [42,43]. MS commonly involves the digestion, ionization, and capture of the proteins by the MS system and later, the detection of the molecules according to the mass-to-charge ratio (m/z) [44]. Mass spectrometers consist of three basic components: an ion source, a mass analyser, and an ion detector. MS measurements are carried out on ionized analytes in the gaseous phase, requiring a method to transfer molecules from solution or solid phase into this state. The two most commonly used techniques are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) [45,46]. Both MALDI and ESI are soft ionization techniques in which ions are created with low internal energies and thus undergo little fragmentation. Electrospray and matrix-assisted laser desorption ionization processes provide ‘soft’ (meaning low-energy) ionization methods for a variety of
bimolecules, including peptides, proteins, drug metabolites, oligonucleotides and carbohydrates, to enable their measurement by MS [47].

There are several mass spectrometers that combine ESI or MALDI with a variety of analysers. In MALDI, samples are co-crystallised with an organic matrix on a metal target. A pulsed laser is used to excite the matrix, which causes rapid thermal heating of the molecules and eventually desorption of ions into the gas phase. Because of the usage of a pulsed laser, MALDI produces packets of ions rather than a continuous beam. This ionization technique tolerates a reasonable amount of impurities in the sample to be analyzed. On the other hand, ESI is based on spraying an electrically charged sample into a gas flow. This ionization technique produces packets of ions rather than a continuous beam. Because of the usage of a pulsed laser, MALDI produces packets of ions rather than a continuous beam. This ionization technique tolerates a reasonable amount of impurities in the sample to be analyzed. ESI is based on spraying an electrically charged sample into a gas flow. This ionization technique produces packets of ions rather than a continuous beam. Because of the usage of a pulsed laser, MALDI produces packets of ions rather than a continuous beam. This ionization technique tolerates a reasonable amount of impurities in the sample to be analyzed. After ionization, the sample reaches the mass analyser, which separates ions by their mass-to-charge ratios. Ion motion in the mass analyser can be manipulated by electric or magnetic fields to direct ions to a detector, which registers the numbers of ions at each individual m/z value.

In proteomics research, four basic kinds of mass analysers are currently being used: time-of-flight (ToF), ion trap, quadrupole (single and triple or tandem), and Fourier transform ion cyclotron resonance (FTICR) analysers. All four differ considerably in sensitivity, resolution, mass accuracy and the possibility to fragment peptide ions [44]. The combination of an ion source, mass analyser and detector is usually determined by the application. The human blood proteome is frequently assessed by protein abundance profiling using tandem mass spectrometry [49].

The use of tandem-MS has demonstrated that Philadelphia chromosome positive (Ph+) B cell expressing IK6 showed increased expression of a set of adhesion molecules, including thymocyte differentiation antigen 1 (THY1, CD90), L-selectin, and THY1 ligand integrin alpha 5, leading to an aggressive lymphoid leukemia phenotype [50]. Interestingly, retinoid treatment abrogates the IK6 phenotype by promoting expression of IKZF1, demonstrating the role of IKZF1 alteration in the leukemogenesis process and its potential therapeutic use for BCP-ALL. In another study, Saha et al., by using 2D-PAGE followed by matrix-assisted laser desorption/ionization time of flight-tandem MS (MALDI-ToF-MS/MS), could identify 79 proteins differentially regulated in CD19+ cells from BCP-ALL patients [51]. Ontology analysis of these proteins demonstrated their participation in proteostasis, cytoskeletal organization, and signal transduction, thus suggesting their potential implications in BCP-ALL leukemogenesis.

When BCP-ALL has already been established, early diagnosis is important for better management and to significantly contribute in the primary induction therapy for the remission of the disease. Cavalcante et al., by using lectin affinity chromatography and liquid chromatography-MS (LC-MS) identified 96 different proteins in serum samples of patients diagnosed with BCP-ALL [52]. Leucine-rich alpha-2-glycoprotein 1, Clusterin, Thrombin, Heparin Cofactor II, Alpha-2-macroglobulin, Alpha-2-antiplasmin, Alpha-1 antitrypsin, and Complement factor B and C3 were over expressed in BCP-ALL in comparison with the induction therapy group, which means that candidate biomarkers only are seen in the disease state and are therefore proposed as potential biomarkers for diagnosis and favorable response after induction therapy [52]. In a similar manner, Mirkowska et al., by using a combination of LC-MS and FC, analyzed the surface protein composition of B-cell precursors of BCP-ALL patients xenografted on immunodeficient mice [53]. They identified 713 surface proteins, including glycosylated and lysine-containing proteins (hematopoietic and B-cell markers). FC validation of some of these molecules (CD63, CD97, CD157, and CD305) in human samples highlighted their potential uses in the diagnosis of BCP-ALL.

Another example of the use of MS in the identification of new molecules for diagnosis and risk stratification in BCP-ALL the study carried out by Xu et al. [54]. Authors analyzed by liquid chromatography-

### Table 2. Examples of potential molecular biomarkers for diagnosis, prognosis, and targeted therapy of BCP-ALL.

| Biomarkers | Functions | Applications in B-ALL | References |
|------------|-----------|-----------------------|------------|
| CD79a      | B-cell activation. | Diagnosis of B-ALL and subtype classification. | [29,35–38] |
| CD58       | Catalyzes the synthesis from cADP ribose to ADP ribose. | It is used for B-ALL diagnosis. | [30–32] |
| TPS3, JAK2 | Tumor suppressor-transcription factor, Participates in intracellular signaling upon activation of cytokine receptors. | Both molecules have been tested as potential biomarker of poor outcome in B-ALL. | [39–41] |
| RAS        | GTPase involved in protein cell signaling. | Unfavorable prognosis and predictive biomarker for treatment. | [42,43] |
| LRG1, CLU, F2, SERPIND1 | Participates in vascularization by modulating TGF-β signaling, Promotes or inhibits tumorigenesis based in the cellular context, Anticoagulant and proinflammatory, Coagulating process. | This cluster of proteins have the potential to be used for B-ALL diagnosis and treatment evaluation. | [52,106,107] |
| CSRP2      | Participates in breast cancer cell invasiveness. | Diagnosis/treatment monitoring for B-ALL. | [47-108] |
| ADA1       | Implicated in purine salvage pathway. | Diagnosis/treatment monitoring for B-ALL. | [46-109] |
| IKZF1, CRLF2 | Participates in the regulation of hematopoiesis, Proto-oncogene in B-ALL. | Both molecules can be used as markers for poor prognosis in high-risk ALL. | [49,50,110,111] |
| Hsp90      | Participates for folding and regulation of cellular proteins. | Diagnosis/treatment monitoring for B-ALL. | [51,52,112,113] |
| A20        | Inhibits activation of NF-κB and apoptosis. | Target for B-ALL treatment. | [53,114] |
### Table 3. Proteomic tools in studies of BCP-ALL.

| Tool                     | Type of study | Objective                                                                 | Findings                                                                 | References |
|--------------------------|---------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------|------------|
| LC-ESI-MS                | In vitro      | To analyze the differential protein expression in the pediatric B-cell line CCRF-SB during vincristine treatment. | Vincristine treatment led to the expression of 135 proteins participating in Toll receptor signaling pathway, Ras pathway, B and T cell activation, CCKR signaling mapping, cytokine-mediated signaling pathway, and oxidative phosphorylation. | [83]       |
| MFC                      | Case-control  | To analyze the expression of B-, myeloid, T-lymphoid and NK-cell antigens in 200 blood or bone marrow samples of pretreated children and adult patients with B-ALL. | Aberrant expression of myeloid-associated antigens was observed in 86.2% of cases and differential expression between children and adult patients with B-ALL. | [33]       |
| MFC                      | Case-control  | To analyze the differential expression of TdT, CD34, CD45, CD10, CD38, CD20, and CD22 in CD10+ and CD10- subpopulations of B-ALL cells and hematogones. | Differential expression was observed between CD10+ in CD10+ subpopulations. CD10+ blasts exhibited higher levels of TdT, CD22, CD34, and CD20. Conversely, CD10- blasts showed higher expression of CD45 than CD10+ blasts. | [32]       |
| MFC                      | Case-control  | To identify the malignant clone harboring clone-specific genomic markers in 53 bone marrow samples derived from 28 children diagnosed with B-ALL for B-ALL MRD monitoring. | FC could identify 93% true-positive (leukemic) and 93% true-negative (normal) cell populations and four discrepant cases for MRD monitoring in B-ALL cases. | [115]      |
| MFC                      | Case-control  | To analyze the immunomodulation of cell surface-antigen in MRD-positive samples of children with B-ALL for MRD detection. | A downregulation of CD10, CD19, and CD34 and upregulation of CD20 were observed after 15 and 33 days of treatment, but no changes in the expression of CD38, CD58 and CD45 were noticed. | [38]       |
| MFC                      | Case-control  | To standardize an immunophenotyping protocol for MRD monitoring and for sensitivity improvement in 319 bone marrow samples of children with B-ALL after induction therapy. | A standardized FC protocol showed a high percentage of concordance (93%) in comparison with RQ-PCR-based MRD detection. | [39]       |
| MFC                      | Case-control  | To standardize a multicolor FC protocol to evaluate the MRD in 263 bone marrow samples derived from childhood relapsed B-ALL. | FC analysis by using a set of B-cell markers showed a consistently high overall concordance (P < .001) and, under optimal conditions, sensitivity comparable to the PCR method for MRD detection. | [40]       |
| MFC                      | Case-control  | To analyze the expression of CD73 in normal young hematogones cells and B-ALL cells with or without MRD+ of bone marrow for MRD monitoring. | CD73 expression was 6-fold greater in MRD-positive B cells in comparison with MRD-negative ones. In all, 41.82% MRD-positive B-ALL cases expressed high CD73 and sensitivity MRD detection reached 10−4. | [116]      |
| MFC                      | Case-control  | To analyze the expression of Neurephilin-1 (NRP-1)/CD304 as an MRD and prognosis marker in bone marrow samples derived from 70 children with B-ALL and 40 controls. | CD304 expression was higher in 40% of B-ALL (CD304 +) cells at diagnosis compared with controls. However, at day 28 of induction therapy, 57.1% of CD304+ patients transitioned to a CD304- phenotype. | [41]       |
| LC-MS/MS                 | Case-control  | To analyze protein expression for risk stratification in BM samples derived from B-ALL patients. The patients were classified into low/medium and high-risk groups. | 86 differently expressed proteins were identified in childhood high-risk ALL patients. They found the aberrant events happened in pre-mlRNA splicing, DNA damage response, and stress response. | [54]       |
| AC-MS                    | Case-control  | To analyze the protein expression profile and biomarker discovery in 10 samples derived from B-ALL patients. | A panel of molecules (i.e. LGI, CLU, F2, SERPIN1, A2M, SERPIN2, SERPIN1A, CFB, and C3) were identified as potential biomarkers for early diagnosis of B-ALL. | [52]       |
| LC-MS/FC                 | In situ/in vivo | To analyze the protein expression profile in immunodeficient mice xenotransplanted with B-ALL human cells. | A total of 713 proteins were detected in B-ALL cases. From all these proteins, CD97, CD157, CD63, and CD305 were identified as the most promising makers to distinguish between normal and malignant cells. | [53]       |
| SELDI-ToF-MS             | Case-control  | To analyze the protein expression profile and biomarker discovery in samples derived from ALL pediatric patients. | FF4, CTP-III, and two fragments of C3a were differentially expressed and used to distinguish pediatric BCP-ALL patients from healthy controls, and pediatric AML patients. | [117]      |
| LC-MS/MS                 | Case-control  | To analyze the role of IKBf1 in B-ALL leukemogenesis. | IKBf1 and Arf alterations contribute to leukemogenesis by promoting the development of an aggressive lymphoid leukemia. | [50]       |
| 2D-PAGE/MALDI-ToF-MS/MS | Case-control  | To analyze protein expression profiles and protein functions in B-ALL patient samples. | A total of 79 differentially regulated proteins were identified in B-ALL cells, participating in proteostasis, cytoskeletal organization, redox homeostasis, and signal transduction pathways relevant to leukemogenesis. | [51]       |
| SDS-PAGE/WB/RPPA         | Case-control  | To analyze the expression and function of Aurora B kinases in 172 B-ALL pediatric patients. | Expression of AURKB and AURKA was mostly observed in E2A-PBX1-translocated B-ALL cases. AURKB inhibition resulted in proliferation arrest and apoptosis. Increased phosphorylation of ErbB2 was observed in 56% of Ph+ ALL. ErbB2 kinase activity inhibition by canertinib resulted in increased expression of the proapoptotic protein Bim, caspase-3 activation, and cell death. | [69] [70] |
| RPPA                     | Case-control/ in vitro | To analyze the protein expression profiles for identification of new therapeutic targets in 129 samples derived from Ph+ B-ALL patients and by using Z-181 and Z-119 cell lines. | CG was overexpressed in B-ALL samples and was proposed as a poor prognostic marker. Endogenous expression analysis demonstrated that CG can also... | [72]       |
tandem MS (LC–MS/MS) the protein expression profile in 12 BM samples of BCP-ALL from patients classified as low/medium and high-risk groups, and 6 patients with non-malignant hematological disorders (non-ALL group). Considering the non-ALL group as reference, authors identified 86 differentially expressed proteins in the high-risk childhood B-ALL group, including Elongation factor 1-alpha 1, Thyromosin alpha-1 and Histone H3.1. Interestingly, a total of 35 of these proteins were predicted to have directive protein–protein interactions and participate in pre-mRNA splicing, DNA damage response and stress response which could explain the high-risk outcome for BCP-ALL.

### 2.2.1. Mass cytometry (CyTOF)

Over time, cytometry methods have been a powerful tool to investigate the hematopoietic system [55]. In the last years, mass cytometry (CyTOF) emerged as a new technology for high-dimensional multi-parameter single cell analysis that overcomes the limitations of conventional flow cytometry [56]. Recently there has been rapid progress in multiparametric single cell proteomic and genomic research. CyTOF is a single cell proteomics platform that combines elemental MS with FC enabling up to 50 parameters per single cell to be measured [57], it has become a revolutionary tool to investigate the hematopoietic system [57,58]. The high number of parameters facilitates analysis of highly complex cell populations and could be extremely useful for the diagnosis of malignant hematologic disorders, monitoring of MRD, and selection of treatment modalities [59]. In CyTOF, the fluorophore-labeled antibodies are replaced by antibodies of isotopically purified heavy metal. The presence of the bound antibodies is detected through the use of inductively
coupled plasma ionization and time-of-flight mass spectrometry (ICP-MS TOF) analysis of the metal ions that were attached to each antibody. The advantage of this approach stems from the ability of ICP-MS to distinguish ions of different atomic weight with less than 1% signal spillover between adjacent masses [59].

CyTOF allows for the measurement of over 40 markers simultaneously without the concern of spectrum overlap. It has enabled the analysis of intact biological specimen without pre-depletion steps or a drop channel. Therefore, we can view the hematopoietic system comprehensively with high-content dimensionality from conventional 2-D flow cytometry plots [55].

The high number of parameters afforded by mass cytometry enables the organization of numerous distinct cell populations within very complex cell samples, while still allowing sufficient additional measurement parameters to characterize functional properties of the different cell populations [59]. High-parameter analysis of malignant diseases, particularly leukemias, is an area in which mass cytometry could be particularly useful in the clinic, and initial experiments have been performed by several research groups [60–64]. These studies highlight a particular strength of high-parameter analysis: the ability to define rare cell types, such as leukemia stem cells, and simultaneously characterize the functional properties of these cells. Table 3 shows additional studies that complement the applications of CyTOF in the diagnosis and prognosis of BCP-ALL.

2.3. Protein arrays for the study and identification of therapeutic targets of BCP-ALL

Protein array is a high-throughput technology based on a set of antigens or antibodies arranged onto solid support for protein identification and functional analysis. There are three different configurations for protein arrays identified as analytical, functional and reversed phase protein array (RPPA) [65]. Unlike analytical and functional arrays, RPPA works by immobilizing the interest samples (e.g. tissue, cell lysates or fractionated cell lysates) onto a solid surface for protein detection by using a set of antibodies [66]. It is possible to screen thousands of interactions in parallel – protein-antibody, protein–protein, protein-drug, enzyme–substrate – as well as providing potential assays for biomarkers. The different types of arrays have applications in clinical diagnostics, which includes detection of antibodies in autoimmune diseases or biomarkers in sera of cancer patients, as well as in target and drug discovery [67,68] (Figure 2).

2.3.1. Reversed phase protein array usefulness in the search of biological targets of BCP-ALL

The identification of biological targets with significance in BCP-ALL is very useful for the development of new therapeutic approaches of disease. RPPA is a powerful tool to achieve this goal. An example is a study carried out by Hartsink-Segers et al. who analyzed the expression of Aurora A (AURKA) and Aurora B kinases (AURKB) by RPPA in combination with SDS-PAGE and western blot [69]. Expression of both kinases was positive in 172 samples derived from BM or PB of pediatric samples BCP-ALL. Even more, in vitro downregulation of AURKB showed anti-proliferative and pro-apoptotic effects, suggesting that AURKB can play an important role in B-ALL leukemogenesis and demonstrating its utility as a possible target for leukemia treatment. Like AURKB, the human epidermal growth factor receptor 2 (ErbB2) kinase has been shown to play an important role in the BCP-ALL pathological process. Irwin et al. studied 129 samples derived from Ph+ BCP-ALL patients by using an RPPA methodology [70] and they found overexpression of ErbB2 in 56% of these patients. In vitro inhibition of ErbB kinase activity in the B-cell leukemic cell lines Z-181 and Z-119 led to a decreased proliferation and increased sensitivity to BRC/ABL TKI treatment. Thus, the ErbB signaling pathway could be an attractive pathway for new therapeutic interventions in Ph+ BCP-ALL.

Another potential target for BCP-ALL is cathepsin G (CG). CG is normally expressed in AML [71], however, its expression in BCP-ALL has been studied by Khan et al. [72]. By performing an RPPA analysis of 130 patients with BCP-ALL, they showed increased expression of CG in a high proportion of the samples; therefore, CG can be a potential target for therapy in BCP-ALL. On the same sense, to unveil the role of signal transducer and activator of transcription 5 (STAT5) in BCP-ALL, Heltemes-Harris et al., by using an RPPA methodology, performed an analysis of 129 samples collected from the blood and/or BM of BCP-ALL patients [73]. The expression of STAT5 was similar in all analyzed samples, but increased phosphorylation of this molecule was observed in a subset of Ph+ BCP-ALL patients. Similarly, Schinnerl et al. [74] analyzed by RPPA a set of PAX5-JAK2+ BCP-ALL samples from BM patients and found increased phosphorylation of STAT5; thus, the fusion protein PAX5-JAK2 could act as an upstream activator of STAT5, which promotes leukemogenesis, and could be used as a therapeutic target for BCP-ALL.

Pre-B cell receptor (pre-BCR) is expressed on the surface of pre-B cell precursors, and its activation has an important role in the clonal propagation of pre-B cells in BM. Thus, alterations in pre-BCR signaling could contribute in the leukemogenesis process. At this respect, van der Veer et al., by using an RPPA methodology demonstrated that pre-BCR signaling is affected by TCF3 gene rearrangement in BCP-ALL [75]. From 19 TCF3-rearranged and 113 non-TCF3 BCP-ALL pediatric samples analyzed by RPPA, the TCF3-rearranged group showed more significant expression of various elements of the pre-BCR...
signaling, including ZAP70, SLP65, Bruton’s tyrosine kinase (BTK), PI3K-p110δ, and IRF4. Corresponding to its role as an effector of BTK, in vitro analysis in MHH-CALL3 cells showed decreased phosphorylation of Erk1/2 and growth and cell viability after treatment of BTK inhibitor Ibrutinib. Therefore, interference with pre-BCR signaling is an excellent candidate for a therapeutic target for TCF3-rearranged BCP-ALL. In a similar study, the proteomic profile of blood and BM from 192 BCP-ALL patients was analyzed by Shojaee et al. [76]. By mean an RPPA methodology, they observed an increased expression of phospho-ERK-T202/Y204, DUSP6, SPRY2 and ETV5 in normal CD19+ BM pre-B cells and BCP-ALL. In vitro, overexpression of DUSP6 led to pre-B cell transformation and colony formation. Conversely, the 2-benzylidene-3-(cyclohexylamino)-1-indanone hydrochloride (BCI)-induced inhibition of DUSP6 produced the hyperactivation of Erk, the increment of ROS levels and cell death, suggesting that the Erk signaling pathway is controlled by negative feedback through SPRY2, ETV5, and DUSP6, and it can represent an interesting target for BCP-ALL treatment.

2.4. RPPA and MS as approaches useful in the understanding of the molecular basis of treatment resistance in BCP-ALL

Leukemic cells can promote mechanisms that enable the development of resistance to leukemia anticancer drugs, thereby leading to relapse and a poor outcome of the disease [77,78]. Thus, the search for strategies against leukemia drug resistance to develop new therapies have been recently revised [79]. Hypoxia is one the mechanism used for leukemic...
cells to promote cell survival and drug resistance during BCP-ALL treatment. The analysis of protein expression of Nalm-6 and Reh B-ALL cells under normoxic conditions by RPPA showed that the treatment of methotrexate (MTX) and prednisolone (PRD) lead to a decreased cell viability Petit et al. [80]. However, hypoxic conditions lead to overregulation of proteins related to drug resistance and deregulation of pro-apoptotic proteins accompanied by the expression of the anti-apoptotic proteins XIAP, Mcl-1, and Bcl-2. Furthermore, cell viability was higher, suggesting a protective role of hypoxia and anti-apoptotic signals in leukemogenesis, regulating cell death and promoting cell survival.

The regulation of the BM microenvironment through the Wnt signaling pathway is another factor intervening in drug resistance affecting the outcome of BCP-ALL [81]. As demonstrated by Yang et al., by mean of RPPA, the analysis of protein expression under treatment of cytarabine (Ara C) lead to an increased expression of 154 proteins related to anti-apoptotic proteins, drug resistance and different members of the Wnt signaling pathway, such as Lef1, c-Myc and CCND8BP1. Furthermore, in vivo blocking of Wnt signaling sensitizes leukemia cells to drug treatment and improved overall survival, demonstrating its potential therapeutic target. Another studied mechanism is mTOR. By mean of activation of the Wnt pathway, mTORC1 regulates the cell cycle and affects the leukemogenesis process. At this respect, Ghazavi et al., by using an RPPA approach analyzed 72 samples derived from ETV6/RUNX1+ BCP-ALL patients [82]. They found that many of these patients showed decreased expression of the glycoprotein CD200 which corresponded to increased expression of PI3 K, Akt kinases and mTOR. Thus, it seems to be possible that, the use of inhibitors for these kinases could represent a viable approach for ETV6/RUNX1+ BCP-ALL treatment.

Vincristine, a vinca alkaloid, interacts with tubulin disrupting microtubule polymerization and favoring the cell cycle arrest in the M phase, (which is followed by induction of apoptosis), is used in several stages of the ALL treatment [83]. In vitro studies performed by Guzmán-Ortiz et al., showed a differential protein expression in the pediatric B-cell line CCRF-SB during gradual exposition to vincristine [83]. Complementary Liquid Chromatography-Electro Spray Ionization-MS (LC-ESI-MS) analysis, demonstrated that vincristine treatment induced the expression of 135 proteins related with Toll receptor signaling, Ras pathway, B and T cell activation, CCKR signaling, cytokine-mediated signaling pathway, and oxidative phosphorylation. In this way, it is logical to think that each of these mechanisms would be contributing to the resistance to pharmacological treatment by leukemic cells and that, in the best of cases they could be the object of targeted therapies.

3. Validation of biomarkers as a prerequisite for its clinical application

MS, RPPA, 2D-PAGE and/or WB have aroused great interest in research and the discovery of new biomarkers for diagnosis, prognosis, monitoring of MRD and for the identification of biological targets for BCP-ALL. However, before proceeding with any clinical application, the confirmation of the usefulness of potential protein markers must be assessed in a prospective or retrospective manner [84]. Once the marker has been approved for clinical practice, its detection and measurement must be rapid and easy to perform, considering quality parameters such as maximum sensitivity and specificity. Unfortunately, such requirements are not entirely filled by the most of the molecular markers reported, and the search for new biomarkers is an arduous task, because it involves the collection of several samples and then, the analysis of thousands of potential molecules (Table 3).

In addition, determination of the biomarker needs to be performed in a readily obtainable specimen (e.g. blood, serum, urine, etc.) and monitored by a non-invasive method. It is desirable that the method is performed on as many samples as possible by using accessible equipment. Consequently, assessment and interpretation of molecular biomarkers are commonly performed through a series of molecular, immunological, or proteomic tools that must be validated for their dynamic range of detection and reproducibility [85].

As for diagnosis and leukemia classification, the identification and validation of biomarkers for BCP-ALL MRD monitoring have been performed routinely by FC. An example of one study in which both the identification and validation of protein markers were carried out, was that performed by Tembhare-Prashant et al. In their study, authors analyzed the expression of CD surface molecules for MRD monitoring in 90 children with B-ALL [86], observed a differential expression of CD24, CD44, CD72, CD73, CD86 and CD200 between hematogones and B-ALL cells after 35 days of induction therapy. In MRD-positive samples, CD73 showed the maximum (83%) frequency of leukemia-associated immunophenotype (LAIP) and CD86, the highest (100%) stability of aberrant expression. Sherif et al., observed that after 14 days of post-induction treatment, there was a reduction in the expression of surface markers CD10, CD19, CD34 and CD97 in 30 pediatric patients diagnosed with BCP-ALL [87]. Interestingly, at day 14, in 81.5% of these patients, CD97 was undetectable. Therefore, the use of these surface markers for MRD monitoring in children with BCP-ALL should be considered.
4. Concluding remarks and challenges

Proteomics has been successfully used to measure the expression of proteins in an organism using samples from different biological compartments. However, the complexity of biological samples increased the possibility of erroneous analysis of molecules. Furthermore, there are not only limitations regarding the performance or applicability of proteomics tools in the investigation of BCP-ALL, but there are also several drawbacks that have their origins in sample or manipulation during its processing, which can be translated into the loss of potential biomarkers for diagnostic or therapeutic purposes.

Since BCP-ALL research based on proteomics tools requires the correct sampling of either the cytosolic or membrane proteins, this will require an adequate extraction method and a suitable reagent that does not interfere with the physicochemical properties of the molecule (i.e. solvent, lysis buffer) [88]. Furthermore, approaches aimed at the search for potential biomarkers derived from blood samples become more complicated, due to the complexity and the enormous abundance of proteins contained in human serum. In this way, one of the main challenges is the elimination of serum albumin, which represents up to 40% of the total protein in the intravascular space [89]. Even when available methods to eliminate, this and other abundant proteins are efficient, the loss of those proteins with less abundance and that represent potential candidates as biomarkers remain as the main challenge.

On the other hand, collection of the specimen being analyzed is of vital importance, and this must be done in the most efficient manner and in the shortest time. Whether the sample is derived from a specific tissue or from blood, these should be collected and following a standardized protocol minimizing errors to obtain real and representative results. For example, some of the variables that may affect the results obtained by blood samples may include the type of additive or anticoagulant used (i.e. heparin, EDTA or sodium citrate), temperature and processing time, hemolysis of the sample, storage temperature of the sample, as well as the processes of freezing and thawing the sample.

Although proteomics offers enormous advantages through the analysis of large amounts of sample, technical obstacles still do not allow its basic or clinical application in a routine way. Moreover, the costs in the proteomic tools and their complexity, the high prices in the consumables as well as trained personnel, represent a real inconvenience at the time of their application. On the other hand, we must point out the enormous challenge represented by the integration of data derived from proteomics research, with those obtained in other branches, such as genomics or metabolomics, which must comply with standardized and valid guidelines.

The translation of a potential biomarker from basic investigation to clinical practice involves a slow and gradual process, which depends mostly on the qualification of the tool and the validation of the biomarker itself. Some of these reasons mean that the identification of potential biomarkers by protein detection-based tools does not yet have the expected impact on decision making within clinical practice. However, the incorporation of bioinformatics and protein databases has facilitated the analysis of information produced by high-throughput proteomic method expanding the use of proteomic tools beyond basic research. In the near future, proteomic tools such as MS, CyTOF, RPPA or FC, could yield better performance as long as established protocols consider, for instance, sample collection and storage, the well-controlled design of case-control studies, and the reduction of costs in their applications. However, it is undeniable that applications of these tools have increased our knowledge of BCP-ALL, and they have had a positive impact on the diagnosis and treatment of this disease.

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