SAPIENZA
UNIVERSITÀ DI ROMA

Department of Molecular Medicine

Sapienza University of Rome

PhD course on Innovation in Immuno-mediated and hematological disorders: innovation in hematology

37° Cycle

Refining measurable residual disease monitoring in Philadelphia-positive acute lymphoblastic leukemia adult patients: new insights from methodological and molecular standpoints

Tutor: Prof. Sabina Chiaretti

Candidate: Dr. Marco Beldinanzi

Coordinator: Prof. Silvano Sozzani

Academic Year 2023 - 2024

Index

ABSTRACT

Measurable residual disease (MRD) negativity represents the primary endpoint in the management of adult Philadelphia-positive acute lymphoblastic leukemia (Ph+ ALL) patients. MRD assays must be highly sensitive and specific and the markers must be reliable and representative of the disease. Droplet digital PCR (ddPCR) and next-generation sequencing (NGS) can overcome some limitations of standard methodologies. In order to evaluate the best strategy for MRD monitoring, in this study were performed: i) *BCR::ABL1*-based MRD monitoring between RT-qPCR and ddPCR; ii) MRD evaluation by *BCR::ABL1* fusion transcript and IG/TR clonal gene rearrangements ; iii) a comparison of the MRD concordance rate between the two markers; iii) a correlation with biologic features and clinical outcome. Samples derived from 111 adults enrolled in the ongoing phase III GIMEMA ALL2820 clinical trial. At diagnosis, patients underwent a screening for the identification of the predominant IG/TR rearrangement and for the *IKZF1*^{plus} signature. Firstly, a *BCR::ABL1*-based comparative study was conducted on 156 MRD samples between the gold standard methodology, i.e. RT-qPCR, and ddPCR showing an optimal correlation degree (R^2 = 0.93) and a concordance of 59.6%.

Ninety-seven/111 (87.4%) cases were evaluable for IG/TR MRD monitoring. At day +70 (end of induction phase), 97 patients were studied and the concordance rate between *BCR::ABL1* and IG/TR MRD monitoring was 46.4%; at day +133 (during consolidation phase), 70 patients were studied and the MRD concordance rate was 41.4%.

Five patients experienced a hematologic relapse and a retrospective backtracking was carried out at a previous time-point: 1 was concordantly *BCR::ABL1*^{pos} and

IG/TR^{pos} , 1 was *BCR::ABL1*^{pos} and IG/TR^{neg}, while the other 2 cases were both *BCR::ABL1*^{neg} but IG/TR^{pos} at 4.0E^{-05} and 2.0E^{-04}, respectively, suggesting the presence at the onset of non Ph+ subclone. Overall, the concordance rate between *BCR::ABL1* and IG/TR is limited, in line with the literature. Nevertheless, a double-hit strategy may be informative for MRD monitoring and possibly for the distinction between typical/lymphoid Ph+ ALL vs CML-like/multilineage Ph+ ALL and, more important, in some cases for predicting hematological relapse.

Finally, a case report of an *IKZF1*^{plus} patient with an extremely aggressive disease was extensively analyzed.

1 Acute lymphoblastic leukemia

1.1 Introduction

Acute lymphoblastic leukemia (ALL) is a hematological malignancy characterized by impaired differentiation, proliferation and accumulation of lymphoid progenitor cells in the bone marrow and/or extramedullary sites. While ALL predominantly affects children, adult ALL presents a more challenging therapeutic approach. ALL exhibits a bimodal distribution: it is primarily regarded as a pediatric leukemia, with 80% of cases occurring in children and 20% occurring in adults[1–3]. It has been observed that there is a peak incidence at 2 to 5 years of age and a subsequent increase after the fifth decade. The median age at diagnosis is 2-10, and approximately 60% of patients are diagnosed at a younger age than 20 years, 25% at around 45 years of age, and 11% at around 65 years of age. ALL is relatively uncommon during late childhood, adolescence, and young adulthood. ALL occurs with an incidence of approximately 1 to 1.5 per 100,000 persons; it is also worth noting that there is a predominance in the male gender (ratio of 2:1)[4]. While the overall survival (OS) for childhood ALL is approximately 85% at 5 years, it is worth noting that the prognosis is still suboptimal in adulthood (approximately 50%), and even worse in older patients (≥ 60 years old), where the 5-year survival rate drops to just 25%[5]. The etiology of ALL is largely unknown. Several genetic factors, most notably Down's syndrome, have been identified as being associated with an increased risk of ALL. However, the majority of patients do not have any recognized inherited factors. Genome-wide association studies have identified polymorphic variants in several genes (including *ARID5B*, *CEBPE*, *GATA3*, and *IKZF1*) that are associated with an increased risk of ALL or specific ALL subtypes[6–8]. Rare germline mutations in *PAX5* and *ETV6* are linked to familial ALL[9,10]. Few environmental risk factors have been identified as being associated with ALL in

children. An elevated incidence of the disease has been associated with exposure to radiation and certain chemicals. However, these associations account for only a negligible proportion of cases[11].

1.2 **Pathogenesis**

ALL comprises multiple distinct entities with varying somatic genetic alterations. It is now known that these abnormalities induce a loss of control exerted by anti-oncogenes, an activation of oncogenes and the formation of new chimeric proteins with oncogenic capacity. ALL is thought to originate from the accumulation and cooperation of multiple genetic lesions that occur in haemopoietic progenitors already heading for differentiation into B- or T-lineage cells, including mutations that confer the capacity for unrestricted self-renewal and those that lead to developmental arrest in a phase-specific way[12] (Figure 1).

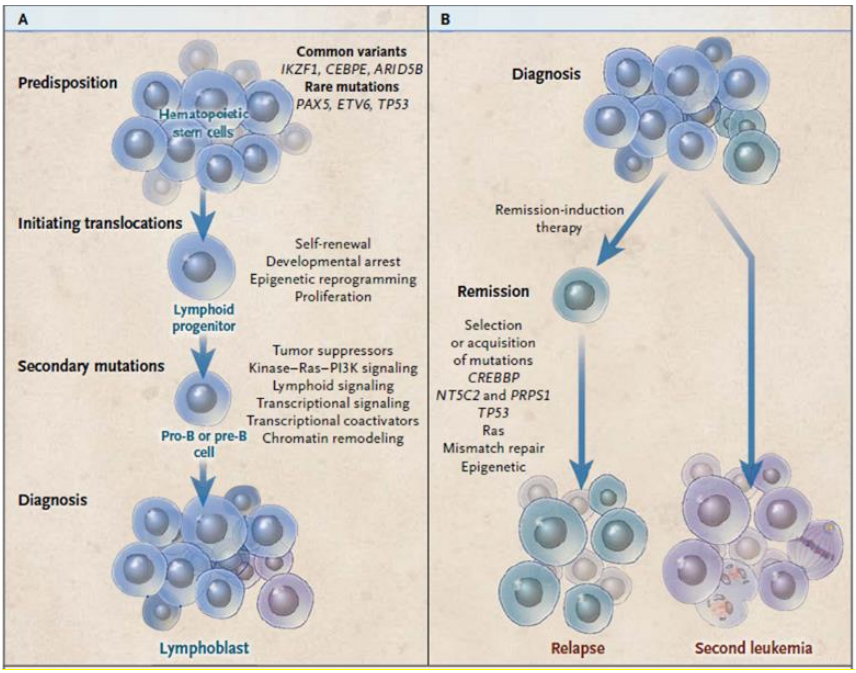


Figure 1 - Proposed Sequential Acquisition of Genetic Alterations Contributing to the Pathogenesis and Relapse of ALL (Hunger SP, Mullighan CG, *N Engl J Med*, 2015).

These alterations include aneuploidy (changes in chromosome number), chromosomal rearrangements that either deregulate gene expression or result in the formation of chimeric fusion proteins, i.e. deletions, gains and sequence mutations at DNA level. At the time of diagnosis, the average number of mutations is approximately 10 to 20; this number increases to approximately 20 to 40 at the time of relapse. Many of these mutations affect key cellular processes, including the transcriptional regulation of lymphoid development and differentiation, cell-cycle regulation, the TP53–retinoblastoma protein tumor-suppressor pathway, growth factor receptor, RAS, phosphatidylinositol 3-kinase, and JAK–STAT signaling, nucleoside metabolism, and epigenetic modification. A perturbation of the two aforementioned processes is commonly observed at the relapse stage.

The characterization of recurrent chromosomal translocations has facilitated the identification of genes that play a critical role in the leukemogenesis process. Gene expression profile (GEP) analysis has been employed to elucidate the distinctions between various genomic/genetic aberrations, thereby reinforcing the hypothesis that specific abnormalities define particular leukemias subtypes[13–15]. These genetic lesions play a crucial role in initiating a leukemic event; however, they are not a sufficient determinant of the complete leukemic phenotype, suggesting that other potentially pathogenic events are necessary[16]. These subtypes are defined by disease-initiating recurrent chromosomal gains and losses, including hyper- and hypodiploidy and complex intrachromosomal amplification of chromosome 21. Additionally, there are subtypes defined by chromosomal rearrangements that deregulate oncogenes or encode chimeric fusion oncoproteins. These rearrangements frequently include cryptic rearrangements that are not identifiable by conventional cytogenetic approaches. Two examples of such subtypes are those defined by *DUX4* and *EPOR*

rearrangements. Finally, single point mutations also define subtypes of this classification (i.e. *PAX5* P80R or *IKZF1* N159Y), subtypes defined by enhancer hijacking (e.g., *BCL11B*-rearrangements in T-ALL and lineage ambiguous leukemia); and subtypes with a similar GEP but different founding alterations (i.e., *BCR-ABL1*-like ALL and *ETV6-RUNX1*-like ALL)[17]. The correct identification of the genetic abnormalities that are responsible for the development of ALL is of great importance for the risk stratification of the disease and the guidance of the incorporation of molecularly targeted therapeutic approaches with the objective of reducing the risk of relapse. Conventional approaches to genetic analysis have included karyotyping, fluorescence in situ hybridization (FISH) and targeted molecular analyses. However, studies conducted over the past decade have highlighted the importance of next generation sequencing (NGS) for the identification of cryptic genetic rearrangements, structural DNA variation and gene expression signatures that were previously undetectable. This has led to a revision of a refined diagnostic approach, whenever possible (Figure 2).

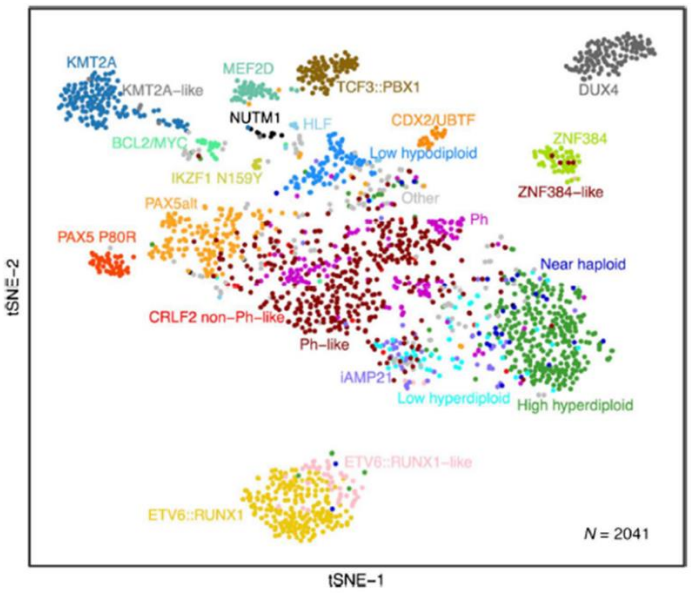


Figure 2 - Gene expression clustering of B-ALL cases (Duffield AS et al, *Virchows Arch*, 2023).

1.3 Laboratory diagnostics and classification

The latest World Health Organization (WHO) classification[18] outlines a multistep approach for diagnosing ALL, which includes the analysis of cytomorphology and cytochemistry, the assessment of immunophenotype, and the evaluation of cytogenetic and molecular characteristics. A comprehensive work-up is essential to facilitate an accurate diagnostic process and precise stratification, as well as to establish the criteria for measurable residual disease (MRD) monitoring. Morphology, multicolor flow cytometry (MFC) and molecular genetics are essential components of the diagnostic work-up. A diagnosis is typically established on the basis of a bone marrow aspirate (BM). In cases where the BM aspirate is compromised and the blast count is elevated, a diagnosis may also be reached through peripheral blood (PB) analysis. Further genomic analyses are conducted to more accurately delineate the molecular subgroups. The material should include nucleic acid (DNA, RNA), viable cells and, if possible, germline material (i.e. saliva collection or oral swab). Subsequently, a lumbar puncture (LP) is conducted, accompanied by the necessary classification and imaging procedures, with the objective of defining potential extramedullary involvement (Figure 3).

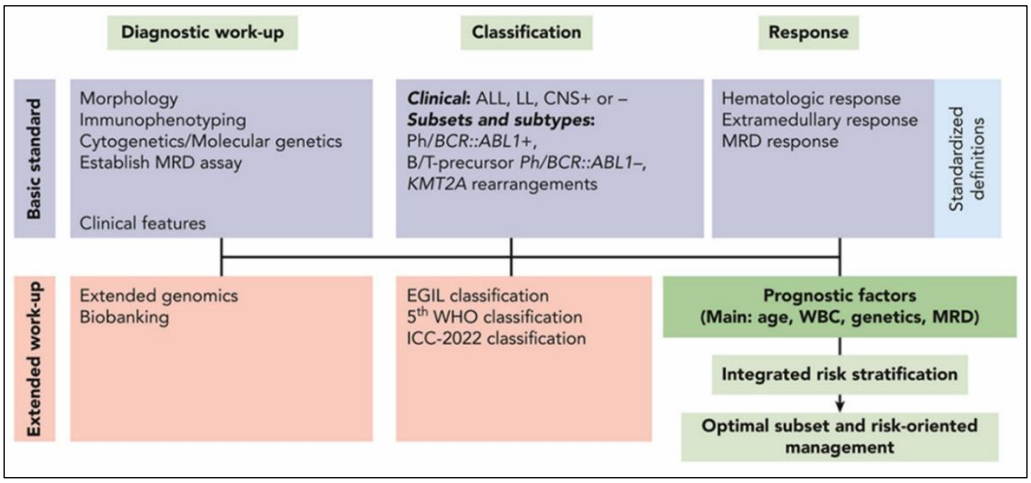


Figure 3 – Basic diagnostic work-up should be performed in patients of all age groups in experienced reference laboratories **(**Gökbuget N et al, *Blood*, 2024)

1.3.1 Morphology and cytochemistry

The initial step in the diagnostic process of an ALL is the observation of BM and PB smears prepared according to the May-Grunwald-Giemsa staining method under an optical microscope. The evaluation of BM morphology is essential for the distinction between ALL and other types of leukemia, including acute myeloid leukemia (AML) and ALL associated with lymphoblastic lymphoma (LBL). There are no specific cytochemical reactions that can be used to classify ALL. Myeloperoxidase (MPO) is consistently negative, except for mixed-phenotype leukemia (MPAL), where low/dim/strong levels of expression have been observed, particularly in B-myeloid cases where isolated MPO expression may occur; on the contrary, in about 95% of cases, the blasts are positive for the nuclear enzyme terminal deoxynucleotidyl transferase (TdT). ALL is defined by the presence of immature elements of the lymphoid line in the bone marrow, previously comprising a proportion equal to or greater than 30% in accordance with the classification proposed by the French-American-British (FAB) Cooperative Group (morphologically distinguishing them into L1, L2 and L3), or 20% in accordance with the WHO[19,20]. Although the morphological subdivision between L1 and L2 forms is no longer employed as it lacks prognostic utility, it remains a valuable tool for the identification of L3 forms, which represent a distinct entity in terms of both biological and clinical characterization, as well as therapeutic treatment and prognosis[21] (Figure 4).

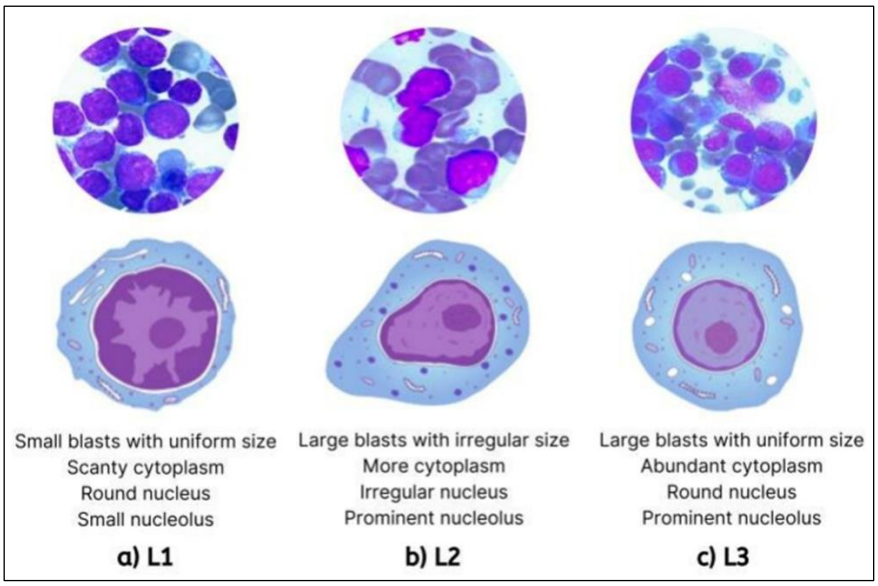


Figure 4 - FAB morphology classification of leukemia cells: A) FAB L1 subtype; B) FAB L2 subtype; C) FAB L3 subtype (adapted from Chiaretti S et al, *Mediterr J Hematol Infect Dis*, 2014).

1.3.2 Immunophenotype

Immunophenotype represents a fundamental stage in the ALL's diagnostic workflow. Indeed, the leukemic cell expresses surface and intracytoplasmic antigens, which are characterized to determine its lineage, level of differentiation and maturation, and the aberrations that identify it. The leukemic blast typically exhibits an immunophenotype that remains stable over time, enabling its detection even when present in a low percentage[22]. The immunophenotyping analysis is conducted using a flow cytofluorometer, which allows for the simultaneous analysis of multiple antigens by employing combinations of monoclonal antibodies (MoAb) conjugated to fluorochromes, allowing for the simultaneous analysis of the antigens expressed, i.e. MFC (at least 8 MoAb may be used in a single analysis). An antigen is considered to be positive on the leukemic cell if it is expressed on at least 20% of cells if surface, or 10% if intracytoplasmic. MFC enables the following: i) the differentiation of ALL from AML, ii) the establishment of lineage affiliation and differentiation, iii) the

definition of an aberrant phenotype for MRD monitoring, and iv) the detection of target antigens for immunotherapy. The EuroFlow consortium has standardized MFC[23] (Table 1):

- **B-lineage ALL (B-ALL)**: B-ALL accounts for 75% - 80% of cases. The most significant markers are CD19, CD22, and cytoplasmic (cy) CD79a. The EGIL[24] classification delineates four distinct differentiation stages: pro-B, B-common, pre-B, and B-mature. Aberrant co-expression of myeloid markers is observed in approximately 40% of cases[25]. Pro-B is $CD10^-$ and frequently associated with t(4;11)/*KMT2A*-r[26], whereas pre-B often carries t(1;19)/*TCF3::PBX1* aberration. Identification of surface markers as potential targets for immunotherapy is crucial for the development of effective therapeutic strategies.
- **T-lineage ALL (T-ALL)**: T-ALL accounts for 20% - 25% of adult ALL. The most significant markers are cyCD3 and sCD7. Furthermore, CD1a, CD2, sCD3, CD4, CD5, and CD8, as well as T-cell receptor α/β or γ/δ, may be variably expressed. T-ALL can be classified into four subtypes: pro-T, pre-T, cortical (thymic) T-ALL and T-mature[24]. Pro–T cases express only cyCD3 and CD7. Cortical T-ALL is CD1a positive, whereas T-mature expresses CD4 and/or CD8, and typically sCD3. The entity designated 'ETP-ALL' (early-T precursor) represents a category within the pro-T/pre-T group that exhibits minimal CD5 expression while simultaneously displaying at least one myeloid and/or stem cell marker[27]. CD34 and myeloid markers are expressed in a proportion of T-ALL cases[25]. The maturation stage correlates with molecular aberrations such as *LMO2::HOXA* in immature, *TLX1::TLX3* in cortical, and *TAL1* in mature T-ALL. There is no uniform international classification of immunophenotypes, other than the EGIL classification.

a.

	Pro-B	B-common	Pre-B	B-mature
TdT	+	+	+	-
CD19	+	+	+	+
cCD79a	+	+	+	+
CD22	+	+	+	+
CD10	-	+	+/-	-
CD20	-	+/-	+/-	+
CD34	+	+	+	+/-
cIgμ	-	-	+	+
sIgμ	-	-	-	+
sk/λ	-	-	-	+

b.

	Pro-T	Pre-T	Cortical-T	T-mature
TdT	+	+	+	+/-
cCD3	+	+	+	+
CD7	+	+	+	+
CD2	-	+	+	+
CD5	-	+	+	+
CD1a	-	-	+	-
sCD3	-	-	+/-	+
CD34	+	+	-	-
α/β or λ/δ	-	-	-	+/-

Table 1 - **a.** B-ALL immunophenotype; **b.** T-ALL immunophenotype.

A small percentage of ALL (1% - 5%) shows the co-expression of lymphoid B/T or myeloid antigens on the same cell - bi-phenotypic leukemia - otherwise on different cell populations - hybrid leukemia[28]. Leukemic blasts can express antigens of multiple lineages simultaneously on a single cell, or alternatively, they can comprise distinct populations of blasts belonging to distinct lineages. The definition of MPAL has been refined in the WHO 2016 classifications. The latter classification system differentiates between B-myeloid and other forms of MPAL[20]. Moreover, the expression of particular non-lineage antigens can facilitate the identification of specific genetic lesions, such as CD66c and CD146 in Ph+ ALL[29].

1.3.3 Cytogenetic analysis and classification

Cytogenetic analysis is a further stage in the characterization of ALL. Conventional cytogenetic analysis has limited capability to identify karyotype alterations in ALL patients, due to the intrinsic difficulty of studying lymphoid blasts in mitosis. New technologies developed on a molecular basis facilitate the identification of previously undetectable karyotype abnormalities. It is crucial to be able to ascertain their presence, as they have been demonstrated to be of prognostic value in the disease. However, the necessity to study representative metaphases of the leukemic clone and the inability to detect minor chromosomal abnormalities underscore the necessity for supplementation with additional tests to detect cryptic cytogenetic abnormalities.

The majority of chromosome abnormalities are structural: the most prevalent are translocations, while inversions, deletions and duplications are less common. As a result of such structural abnormalities, the following consequences may occur: (i) a loss of control exerted by anti-oncogenes, (ii) the activation of oncogenes, and (iii) the activation of new proteins with potential transcriptional activity. Abnormalities in number are less prevalent. The incidence of hyperdiploidy in adults is about 5-10%, and the correlation with a more favorable prognosis is less pronounced than in pediatric cases (present in approximately 25% of cases). In contrast, the presence of hypodiploidy (2-4%) is associated with a poor prognosis in both pediatric and adult patients. Recent studies have demonstrated that cases of hypodiploidy are characterized by specific genetic lesions. With regard to structural alterations, chromosomal translocations are the most prevalent in ALL, occurring in approximately 40% of cases. In particular, regarding the B-ALL setting, as reported in the latest "International Consensus Classification of Myeloid Neoplasms and Acute Leukemias"[30] the most relevant entities with structural genomic aberrations with prognostic significance are as follows:

- **t(9;22)(q34.1;q11.2)/*BCR::ABL1***: characterized by the formation of the Philadelphia-chromosome, which encodes the BCR::ABL1 fusion protein, causing constitutive tyrosine kinase (TK) activation. This chromosomal translocation represents the most frequent aberration in adults, occurring in approximately 25% of cases, with an increased frequency with age.

  Ph-positive ALL (Ph+ ALL) from the prior classification can now be divided into two biologically distinct subsets: lymphoid (or typical) and multilineage (or CML-like). It appears that one of these subsets may be more closely related to chronic myeloid leukemia (CML) presenting in lymphoid blast phase. It should be noted that these subsets cannot be distinguished by the use of p190 vs p210 fusion proteins. However, they can be distinguished by fluorescence in situ hybridization (FISH). This depends on whether the translocation can be detected also in other cellular lineages (i.e. myeloid cells, T-cells, healthy B-cells). Notably, the prognosis and optimal treatment of these 2 variants may differ[31–33].
- **t(v;11q23.3)/*KMT2A*-rearranged**: rearrangements of the mixed-lineage leukemia 1 (*MLL1* or *KMT2A*) gene, on chromosome 11q23 to over 80 different partner genes have been identified as a subtype of leukemia with both lymphoid and myeloid features, as well as an unfavorable prognosis. This subtype of leukemia is predominantly diagnosed in infants (~80%), with a secondary peak of onset in adulthood, where the most common partner of rearrangement is *AFF1*. It is frequently associated with a pro-B immunophenotype and the expression of myeloid markers[34].
- **t(12;21)(p13.2;q22.1)/*ETV6::RUNX1***: represents the most common cytogenetic alteration in childhood ALL (25%) with a frequency <5% in adults. The *ETV6::RUNX1* fusion is regarded as a leukemia-initiating alteration that originates in utero, as evidenced by its detection in

umbilical cord blood and the prenatal monoclonal origin observed in identical twins. The prolonged latency period from birth to the clinical manifestation of leukemia suggests that *ETV6::RUNX1* requires the involvement of additional genetic events to induce leukemia[35].

- **t(1;19)(q23.3;p13.3)/*TCF3::PBX1*:** the t(1;19)(q23;p13) translocation encoding *TCF3::PBX1* accounts for 5–6% of cases, but only 1% of adult cases[17]. This fusion is associated with a pre-B immunophenotype and expression of cytoplasmic immunoglobulin heavy chain, as well as a higher peripheral blood white cell count at diagnosis. Furthermore, it is associated with expression of stem cell and myeloid markers, alterations of *PAX5* (deletions) and the *RAS* pathway[36].
- ***BCR::ABL1*–like (or Ph-like):** Ph-like entity is constituted by cases that have a GEP similar to Ph+ ALL but lack the "true" *BCR::ABL1* translocation[13,14,37]. Further investigation has revealed that these cases exhibiting a Ph-like profile harbor a heterogeneous spectrum of genetic lesions. In order to incorporate these new data, the ICC has divided the category of Ph-like into three distinct subtypes. The *ABL1*-class rearranged, *JAK-STAT* activated, and not otherwise specified (NOS) category. A comprehensive identification of the kinase signaling-activating drivers of Ph-like necessitates genetic studies, as none of these entities, with the exception of rearrangement of *CRLF2* in the *JAK-STAT* subgroup, exhibits distinctive immunophenotypic abnormalities. While Ph-like has an overall poor prognosis, there is some variability based on the exact genetic lesion. The identification of *ABL1*-class fusions in Ph-like is of particular importance, as this subgroup may respond to tyrosine kinase inhibitors (TKIs) that target *ABL1*, such as imatinib, dasatinib, and ponatinib[38]. The *ABL1*-class rearrangements comprise fusions between

ABL1, *ABL2*, *CSF1R*, or *PDGFRB* and assorted partner genes. The rearrangement of these kinases can be identified through FISH, while the specific fusions can be discerned through commercially available targeted transcriptome sequencing, namely RNA-sequencing. The second novel subclassification of Ph-like in the ICC comprises cases that are activated via the *JAK-STAT* pathway. The spectrum of mutations that result in the activation of the *JAK-STAT* signaling pathway is broad: the most prevalent are *CRLF2* rearrangements (*CRLF2*-r) to *IGH* or *P2RY8*, which result in *CRLF2* overexpression. The less common *JAK-STAT* activating alterations include *JAK2* fusions, truncating and activating rearrangements of *EPOR*, and, less commonly, *IL7R* or *CRLF2* mutations. Furthermore, approximately half of *CRLF2*-r cases exhibit mutations in *JAK1*, *JAK2*, or *JAK3*, while *RAS* pathway genes (*KRAS*, *NRAS*, *PTPN11*, and *NF1*) are also frequently mutated in the context of *CRLF2*-r. Finally, the Ph-like NOS subgroup is characterized by a heterogeneous range of alterations affecting other kinases and cytokine receptors. However, it is notable that a significant proportion of these have been demonstrated to respond to TKIs in preclinical models, particularly those involving kinase rearrangements (such as those observed in *FLT3*, *FGFR1*, *NTRK3* and *PTK2B*). Additionally, *RAS* pathway mutations have been observed.

- **B-ALLs with gross chromosomal abnormalities (hyperdiploidy, low-hypodiploid, near-haploid):** hyperdiploidy (>46 chromosomes) has been documented in about 5-10% of adult cases. However, the association with a more favorable prognosis is less pronounced than in pediatric cases, where it is observed in approximately 25% of cases. Low hypodiploidy is more prevalent in adults and is frequently associated with *IKZF2* deletions and *TP53* mutations. Approximately half of the *TP53* mutations observed

in children with low hypodiploid B- ALL are germline, indicating that some of these leukemias may arise in the context of Li-Fraumeni syndrome. Near-haploid B-ALL is more prevalent in pediatric patients and is also associated with a poor prognosis. Despite the markedly distinct prognosis of near-haploid B-ALL and high hyperdiploid B-ALL, they exhibit shared GEP and concomitant mutations, indicating a potential common developmental origin[39].

The ICC currently reports 9 novel entities that were not included in the 2016 WHO classification. These entities are defined by either chromosomal rearrangements or recurrent sequence mutations. The following section will focus on those with the greatest frequency and clinical impact:

- ***DUX4*-rearrangements:** *DUX4*-rearrangement is relatively common in adolescents and young adults (AYA). This entity is associated with an excellent prognosis in both children and adults, even in cases where there are otherwise poor-risk genetic features present, such as *IKZF1* deletion. Furthermore, this prognosis remains favorable despite elevated levels of MRD observed early in therapy. *IGH* is the most common rearrangement partner of *DUX4*[40].
- ***ZNF384*-rearrangements:** *ZNF384* rearrangements define a subtype that is most detected in pediatric and young adult patients[41], with a variable prognosis[42]. *ZNF384* has numerous fusion partners, though *EP300*, *TCF3* and *TAF15* are the most common. The prognosis of *ZNF384*-r B-ALL is dependent on the fusion partner, with *EP300* fusions having the most favorable prognosis[43] and *TCF3* fusions having the worst prognosis.
- ***MEF2D*-rearrangements:** *MEF2D*-rearrangements involve the engagement of disparate genes, including *BCL9*, *HNRNPUL1*, *SS18*, *FOXJ2*, *CSF1R*, and *DAZAP1* and are observed in approximately 6 - 7% of

adult patients and about 3% of pediatric patients. The deregulation of this transcription factor has been observed to result in overexpression of *HDAC9*, which suggests that *HDAC* inhibitors may have therapeutic potential in this particular entity[44].

- ***MYC*-rearrangements:** approximately 4% of adult B-ALL and, infrequently, pediatric B-ALL cases present with *MYC* translocation, which have been associated with a particularly unfavorable prognosis. Previous research examining a limited number of *IGH::MYC*-positive B-ALL cases revealed that the *IGH::MYC* translocation observed in these acute leukemias arises from aberrant V(D)J joining in a B-cell precursor undergoing V(D)J recombination[45].

In conclusion, some provisional entities characterized by hot-spot point mutation have been introduced, of which the most relevant are *IKZF1* N159Y[40] and *PAX5* P80R[46]. The first is rare and produces a missense mutation leading to the upregulation of several oncogenic genes, while the second appears more common, especially in adults, and has a relatively favorable prognosis. A comprehensive overview is shown in figure 5.

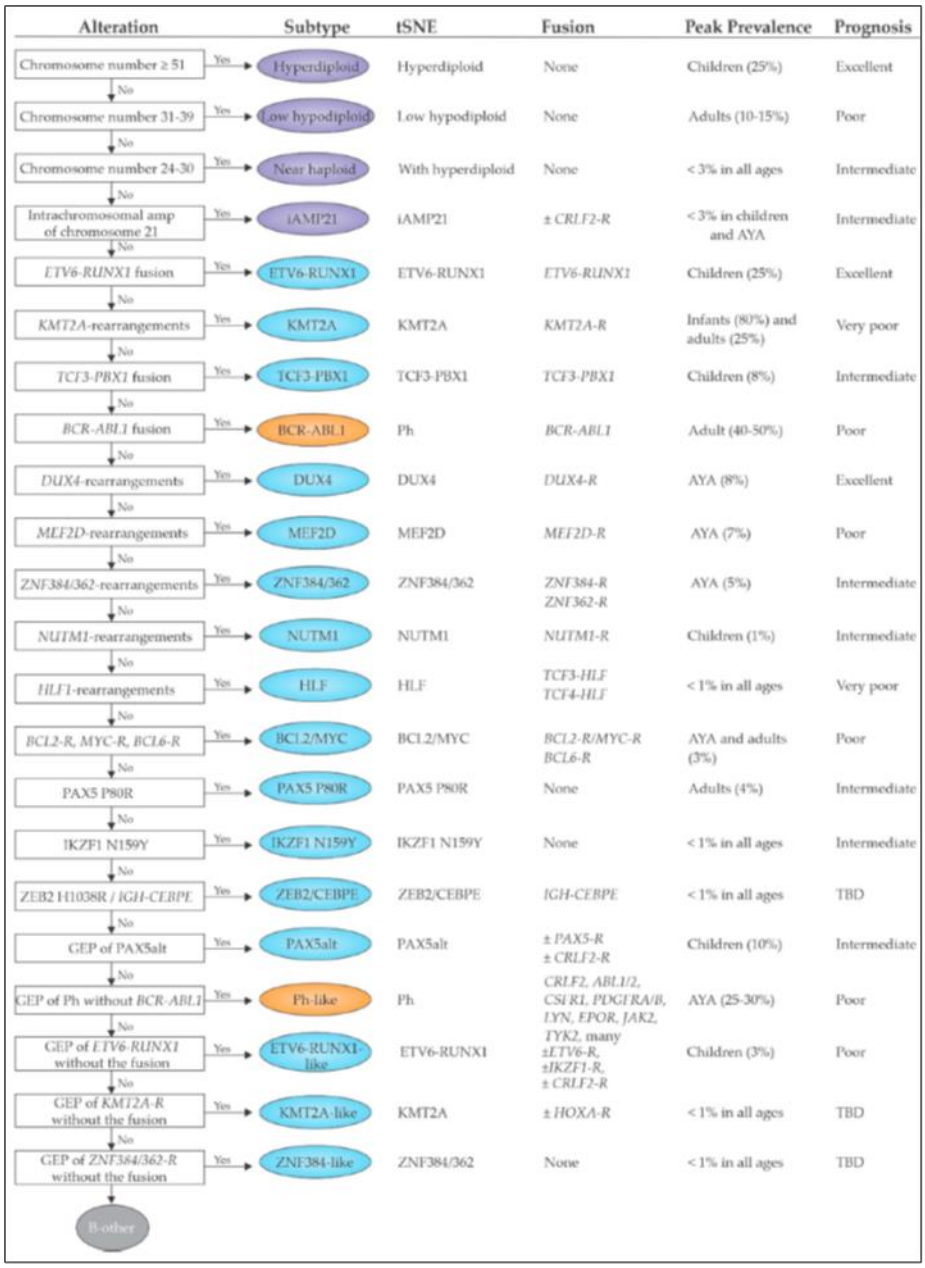


Figure 5 - B-ALL subtype according to the specific genetic alterations (Iacobucci I et al, *J Clin Med*, 2021).

2 Philadelphia-chromosome positive B-ALL (Ph+ ALL)

Ph+ ALL was regarded as the most malignant form of leukemia, with poor prognosis in both pediatric and adult settings due to the limited efficacy of conventional multi-agent chemotherapy[47]. Allogeneic stem cell transplantation (allo-HSCT) represented the sole potential cure, however this was only feasible in a minority of patients due to advanced age and poor response to conventional treatment[48]. The situation changed when TKIs were employed in the treatment of Ph+ ALL, which had previously been confined to CML[49]. Although rare in children with an incidence of about 2 - 5%, this entity represents the most common ALL genetic subgroup in adults, with an overall incidence of 20 - 25%. The incidence increases with age and accounts for more than 50% of cases of ALL in patients who are older than 50 years of age[50] (Figure 6).

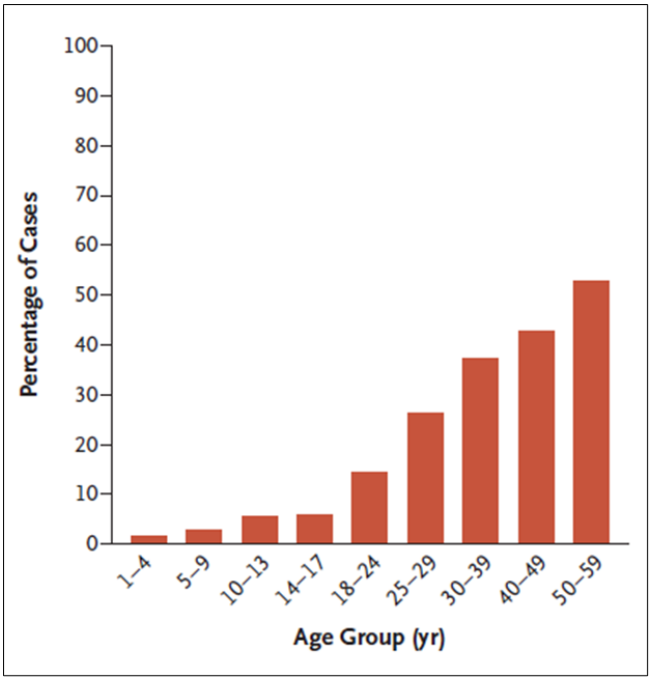


Figure 6 - Incidence of the *BCR::ABL1* rearrangement in Ph+ ALL according to age group. (Foà R and Chiaretti S, *N Engl J Med*, 2022).

The Philadelphia chromosome is the result of a balanced reciprocal translocation combining the proto-oncogene *ABL1* on chromosome 9 with the breakpoint cluster (*BCR*) region sequence on chromosome 22 [t(9;22)(q34.1;q11.2)]. This results in the formation of the BCR::ABL1 fusion protein, which is a constitutively active form of the ABL1 TK. The distinction between the two main types of fusion transcripts is dependent on the breakpoint of the BCR region[48]. The location of the *BCR* and *ABL1* genomic breakpoints is highly variable; however, the recombination process typically involves the fusion of intron 1, intron 13/14, or exon 19 of *BCR* with a 140-kb region of *ABL1* situated between exons 1b and 2. The fusion of *BCR* exon 13 and *ABL1* exon 2 (e13a2) or e14a2 gives rise to a 210 KDa protein, i.e p210 isoform, and constitutes the major *BCR::ABL1* isoform (M-*BCR::ABL1*), which was originally designated as b2a2 and b3a2[52] (Figure 7).

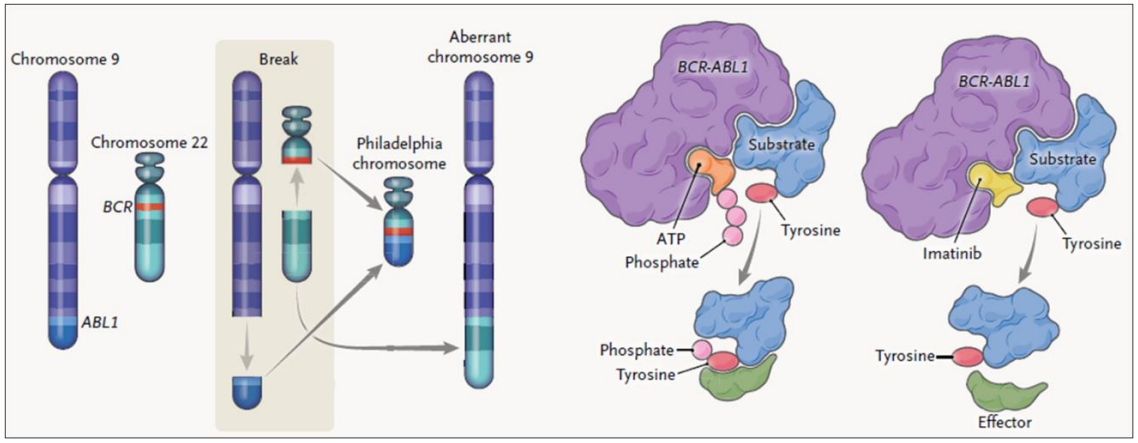


Figure 7 – *BCR::ABL1* structure at genomic and protein levels. (Foà R and Chiaretti S, *N Engl J Med*, 2022).

The p210 transcript is most commonly detected in CML and occasionally in about 30% of Ph+ ALL or very rarely acute myeloid leukemia (AML). The p190 (e1a2) transcript constitutes the minor *BCR::ABL1* isoform (m-*BCR::ABL1*), which encodes a hybrid 190-kDa protein that represents the most typical isoform of the

Ph+ ALL and sporadically in AML, though it is rarely observed in CML. Finally, the p230 (e19a2) transcript, also designated as the μ- BCR::ABL1 isoform (μ-*BCR::ABL1*), encodes a hybrid 230-kDa protein[53]. The transforming activity of *BCR::ABL1* can be attributed to its constitutive TK activity, which plays a role in maintaining cell proliferation, inhibiting differentiation and promoting resistance to apoptosis. *BCR::ABL1* hyperactivity leads to the activation of signaling pathways and to the deregulation of cellular processes. The majority of these pathways have been demonstrated in CML and ALL mouse models. Furthermore, it inhibits the differentiation of hematopoietic progenitors, thereby exerting a pivotal influence on the pathogenesis of leukemia[54]. The main pathways associated with *BCR::ABL1* activity are presented in figure 8.

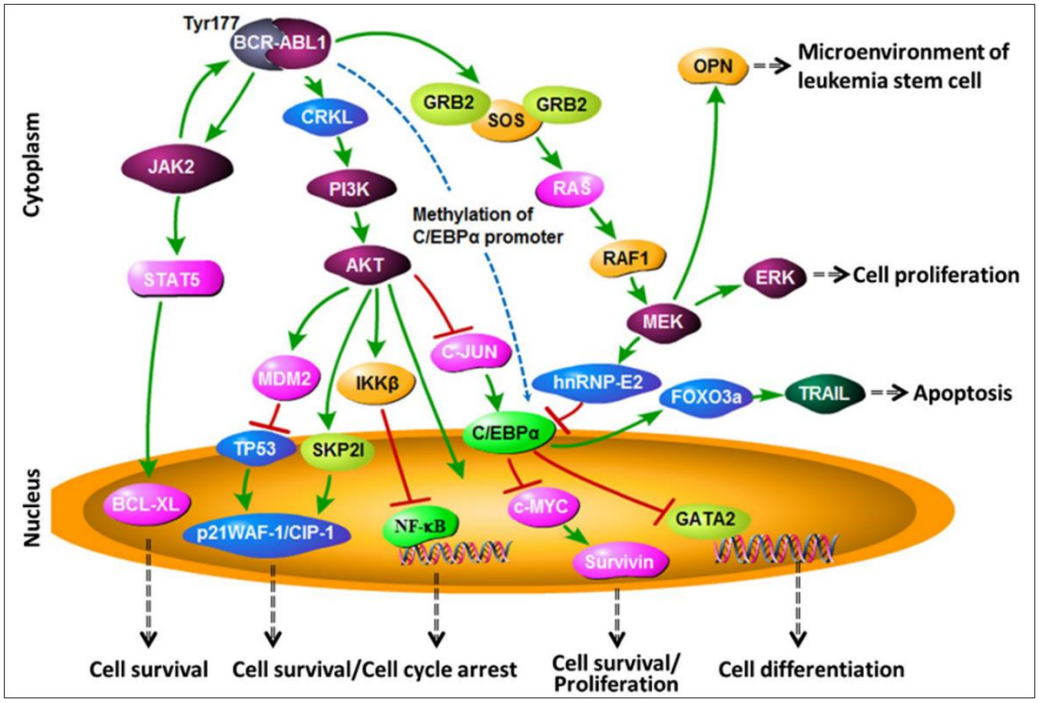


Figure 8 – Main pathways regulated by the BCR::ABL1 protein. (Kang ZJ et al, *Chin J Cancer*, 2016).

Although the *BCR::ABL1* has been identified as the main driver of Ph+ ALL, it is now established that other factors are involved in the disease's development and

progression. Genomic instability has been shown to be an essential factor in tumor formation in both leukemias and solid tumors; in fact, *BCR::ABL1* positive cells have an increased prevalence of double-strand DNA breaks and demonstrate enhanced activity of the single-strand annealing repair pathway[55]. In this context, an extremely important role is played by the *IKAROS* (*IKZF1*) gene, located on chromosome 7p12 that plays a pivotal role in regulating lymphocyte differentiation. The wild-type (wt) form of *IKZF1* has been demonstrated to prevent the acquisition of stem cell-like properties and to exert tumor-suppressor activity in the context of *BCR::ABL1*-initiated leukemia[56]. *BCR::ABL1* and *IKZF1* mutations are strongly linked: a significant proportion of Ph+ ALL cases (70-80%) exhibit *IKZF1* lesions, which are linked to a poor prognosis, high risk of relapse, and a higher chance of therapeutic failure[57] (Figure 9). These deletions can be found mostly in cases with *BCR::ABL1* rearrangements and in the Ph-like subgroup, while its prognostic role in Ph-negative B-ALL is still debating. In particular, the value of *IKZF1* appears to be contingent upon the co-presence of other deletions with a negative prognostic impact, such as those of *CDKN2A/B* and/or *PAX5* genes, condition that defines the presence of the so-called *IKZF1*^{plus} signature[58]. This peculiar copy number variations (CNVs) profile both in pediatric and adult Ph+ and Ph-like ALL patients is associated to a worst outcome and an increased risk of relapse, probably because it resembles a global genomic instability. The *IKZF1*^{plus} was first identified by Single Nucleotide Polymorphism (SNP) Array and since its striking prognostic relevance is defined, new technologies for the CNVs identification have been incorporated in the clinical practice, of particular importance are Multiplex Ligation-dependent Probe Amplification (MLPA) technique and the subsequent evolution the digital-MLPA (d-MLPA) that incorporates the NGS technology.

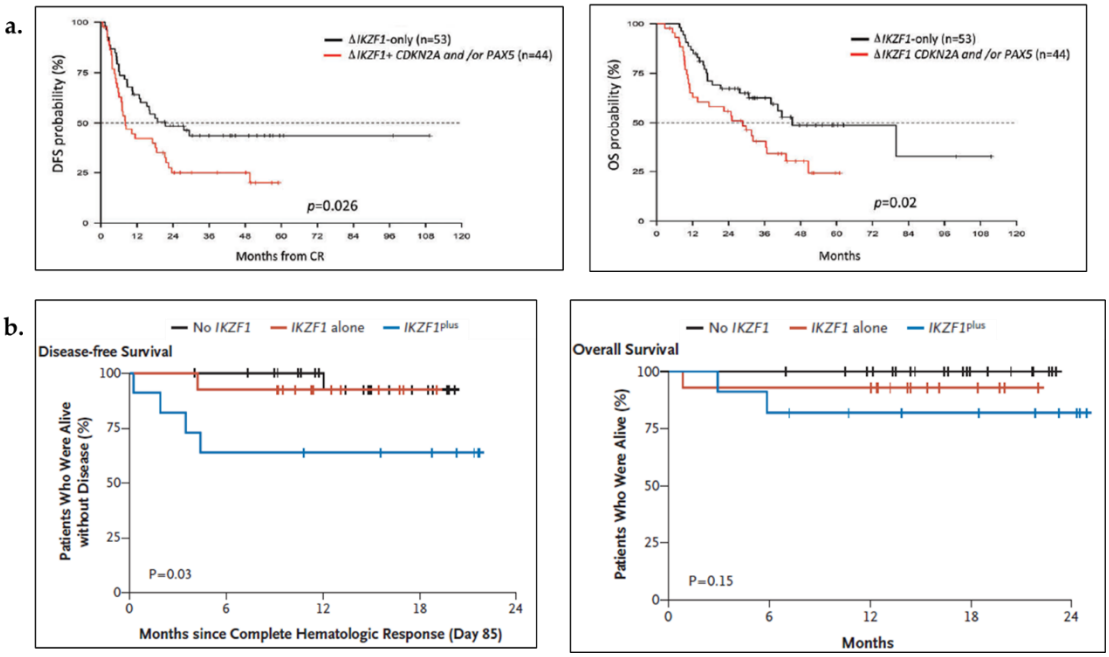


Figure 9 – DFS and OS according to the presence or absence of *IKZF1* or *IKZF1*^{plus} deletions (adapted from: **a.** Fedullo AL et al, *Hematologica*, 2019; **b.** Foà R et al, *N Engl J Med*, 2020)

The diagnosis of Ph+ ALL must be made as quickly as possible due to the advent of new therapeutic strategies. Indeed, the diagnostic workflow is currently made using molecular biology techniques, such as reverse transcriptase polymerase chain reaction (RT-qPCR) and quantitative PCR (RQ-PCR), which allow a response within 24-72 hours. However, conventional cytogenetics remains a valuable tool for identifying the presence of additional chromosomal abnormalities, given their prognostic value.

Ph+ ALL are typically distinguished by a higher white blood cell (WBC) count at diagnosis compared to Ph-negative ALL. However, the clinical manifestations and symptoms are identical and result from the proliferation of leukemic cells in the BM and spreading in PB, as well as the potential damage that these cells may inflict on other organs. From a biological standpoint, it is crucial to conduct a comprehensive immunological assessment and a cytogenetic and molecular investigation. The analysis of the immunophenotype by flow cytometry is

mandatory to show the presence of B-lymphoid line precursors with expression of the surface antigens CD19, CD10 and CD34 (in a high percentage of cases), with frequent co-expression of myeloid markers such as CD13 and/or CD33. In a fair percentage of cases, positivity for the CD66c can also be detected[59].

2.1 Treatment and prognosis

The general backbone of an ALL treatment scheme comprised several distinct but interconnected phases. These include a steroid pre-phase, an induction phase, a consolidation phase, a *sine-die* maintenance phase, and a central nervous system (CNS) prophylaxis phase. The objective of this sequential approach is to eradicate leukemic cells from the bone marrow while preserving normal progenitors. The objective of induction therapy is to achieve complete remission (CR) with the eradication of leukemic cells in the bone marrow, followed by consolidation therapy to eliminate any residual leukemic cells remaining after induction therapy. Finally, a maintenance phase is recommended to limit the risk of relapse[60,61]. Historically, the prognosis for this group of patients has been regarded as unfavorable, with Ph+ ALL being considered the most lethal disease in hematology. Recently, the use of first- or second-generation TKIs inhibitors has not only resulted in an increase in CR, but has also led to a significant shift in the therapeutic approach to patients; we may therefore distinguish two eras, pre- and post-TKIs[62–64].

Imatinib was the first TKI to be tested in clinical trials (1st generation TKIs). Imatinib mesylate is a 2-phenylaminopyrimidine derivative that binds to the activation loop of the *ABL1* kinase outside of a highly conserved ATP-binding site which traps the kinase in an inactive conformation. Imatinib was first tested in the CML setting, where was able to induce a complete cytogenetic response in more than 80% of patients in chronic phase[65,66]. These results highlighted the

potential of molecularly targeted anticancer therapies and led to the widespread development and use of such agents in the treatment of tumors. A recurring theme in resistance to *ABL1* TKI therapy is the development of point mutations within the kinase domain of *BCR::ABL1*. The frequency of *BCR::ABL1* mutations in imatinib-resistant patients ranges from 40 to 90%. To date, more than 50 different point mutations encoding single amino acid substitutions in the kinase domain of the *BCR::ABL1* gene have been identified in patients with imatinib-resistant leukemia[67,68]. At the protein level, these point mutations lead to distorted configurations of the *ABL1* kinase-imatinib interface. Therefore, *ABL1* cannot adopt its inactive conformation where imatinib binds[69]. The impaired interaction between imatinib and *ABL1* kinase is best exemplified by the T315I mutation: this threonine residue at position 315, also known as the gatekeeper, is located near the *ABL1* catalytic domain in the center of the imatinib binding site, which controls access to a hydrophobic region of the enzymatic active site. The T315I mutation poses a major therapeutic challenge and has prompted the need for new strategies to treat imatinib-resistant patients. This has stimulated considerable efforts to develop new generations of TKIs[70,71].

The combination of TKIs with standard-dose chemotherapy and allo-HSCT in fit patients has been shown to improve outcomes compared with historical controls[72]. The next step was to reduce the intensity of chemotherapy. The results of several trials with imatinib or dasatinib have shown that this strategy provides an identical response rate with less toxicity, allowing allo-HSCT to be performed in patients who are in a better condition[73]. The achievement of a complete molecular response (CMR) at 10-12 weeks after initiation of therapy has emerged as an important prognostic factor in patients treated with TKIs and intensive or attenuated therapy and is one of the main goals of treatment. Clinical research has concentrated on the identification of TKIs that can overcome the resistance

induced by *ABL1* mutations and the subsequent reduction in the necessity for chemotherapy. The objective was to develop chemotherapy-free therapies by incorporating novel agents into the therapeutic regimen, with monoclonal antibodies representing a significant advancement in this regard[74]. In this context, the GIMEMA (Gruppo Italiano Malattie EMatologie dell'Adulto) LAL2116 (D-ALBA) clinical trial represents a milestone[75]. D-ALBA was a phase 2 study evaluating frontline treatment of Ph+ ALL in adults with no upper age limit. The therapeutic scheme was chemotherapy-free and comprised the administration of the second-generation TKI dasatinib in association with glucocorticoids during the induction phase, followed by at least two cycles of the bispecific monoclonal antibody blinatumomab in the consolidation phase. Briefly, blinatumomab targets CD19, an antigen presents on virtually all B-lineage ALL, and CD3, which is present on all T-cells and exerts its anti-leukemic effect by inducing an immunological synapsis between the leukemic blasts and the host T-cells, inducing the killing of the leukemic blasts. At the end of the induction phase, 98% of patients had a hematological remission and 29% had a molecular response. Overall survival (OS) and disease-free survival (DFS) rates at 18 months were 95% and 88%, respectively. In a recently published update at a median follow-up of 53 months, DFS, OS, and event-free survival (EFS) are 75.8%, 80.7%, and 74.6%, respectively, with no events in the early responders[76] (Figure 10).

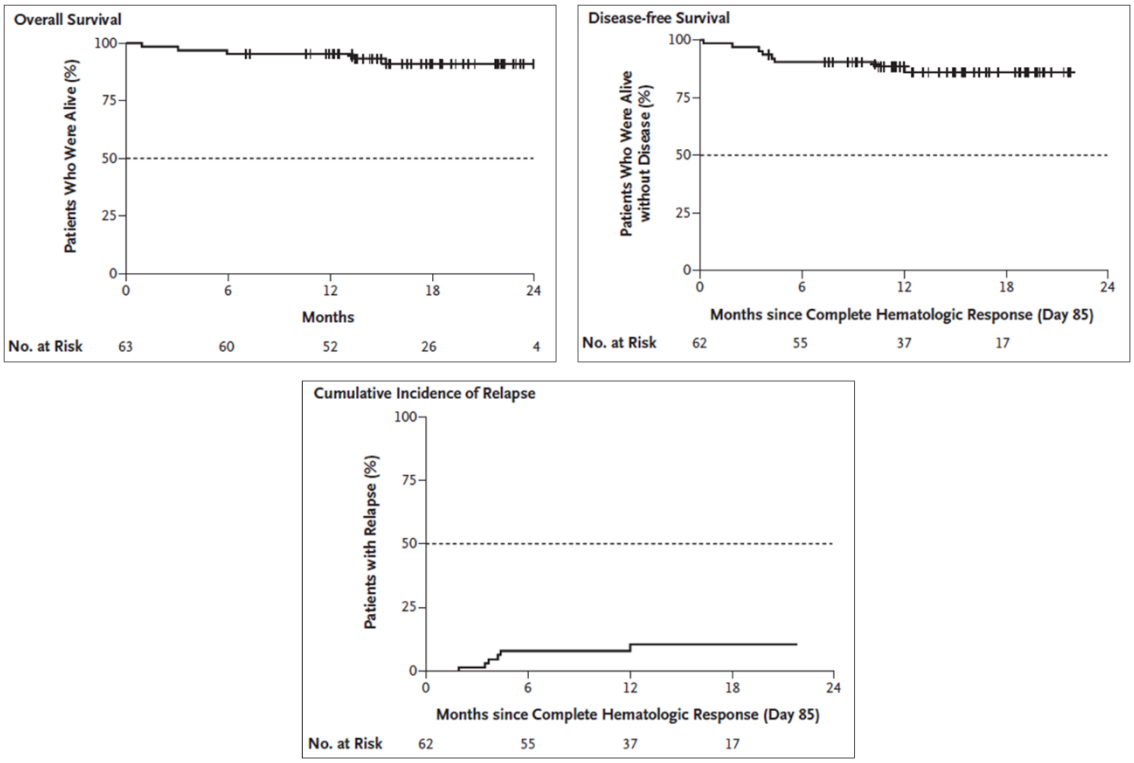


Figure 10 – OS, DFS, and cumulative incidence of relapse in the GIMEMA LAL2116. (Foà R et al, *N Engl J Med*, 2020).

Despite substantial improvements in the outcome of Ph+ ALL and the use of current first-line strategies with TKIs in combination with immunotherapy, hematological relapse occurs in about a quarter of patients, making their management challenging. The optimal current approach involves the adjustment of TKI according to the mutation profile at relapse and the integration of immunotherapy, if feasible, given that immunotherapeutic strategies are not universally accessible[77]. A subsequent allo-HSCT should be considered, if not already performed, or Chimeric Antigens Receptor Cells-T (CAR-T) therapy should be considered in case of relapse following a transplant[78]. Another emerging issue is represented by CNS relapse[79]. Prophylaxis of CNS is initiated during the induction phase and continued throughout the treatment phases[80]. There is currently no clear strategy for treating CNS relapse; however, there is a tendency to increase the number of prophylactic lumbar punctures to avoid it.

2.2 Measurable residual disease (MRD): clinical and methodological insights

MRD describes the proportion of malignant cells that remain in the BM or, less frequently, in the circulating blood after treatment. In general, ALL is the first neoplasm where the assessment of early response to therapy by MRD monitoring has proven to be a pivotal tool for guiding therapeutic choices. Moreover, MRD plays a key role in prognosis and treatment decisions and the achievement of high rate of MRD eradication represents a primary aim, therefore MRD assays are required to be highly sensitive ($\geq 10^{-4}$) and to have broad applicability, accuracy, and reliability[81]. The molecular biomarkers should be representative of the disease, specifically expressed by leukemic clones and stable overtime. Several studies have employed either flow cytometry or a molecular approach at various time points (TPs) during treatment to assess MRD levels. This has enabled the establishment of MRD cutoff values, which have been used to categorize patients into different risk-adapted treatment groups. Additionally, MRD is employed to monitor disease burden in the context of allo-HSCT, providing an early indicator of potential relapse at any stage of the disease[82–84]. Basically, there are two parameters that influence the type of approach used to monitor the MRD: the sensitivity, i.e. the type, of the methodology and the specificity of the biomarker (or marker) used (Figure 11).

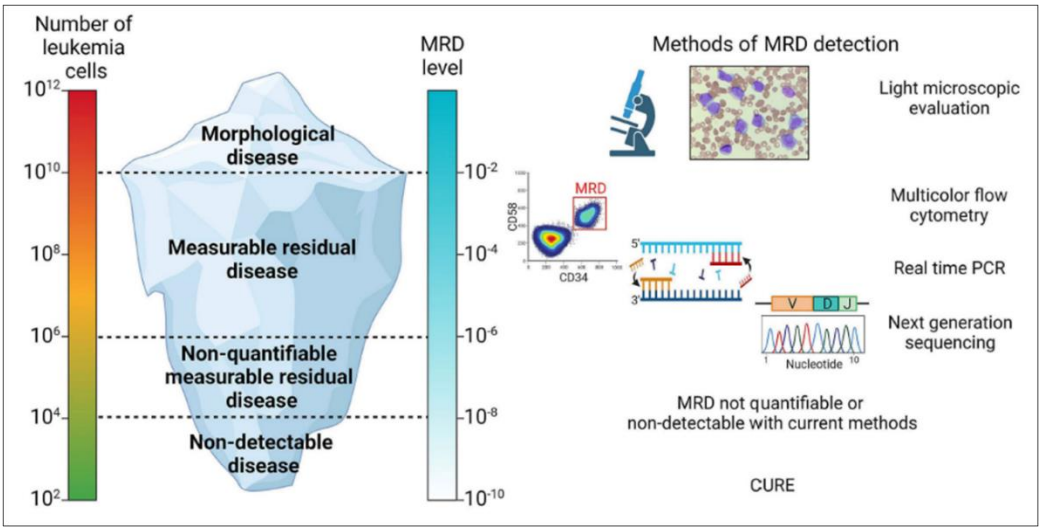


Figure 11 – MRD assessment in ALL: schematic representation of disease levels with corresponding methodology for the monitoring (Saygin C et al, *Hematologica,* 2022).

Currently, the most standardized molecular methods to study MRD are polymerase chain reaction (PCR)-based methods that use leukemia-specific or patient-specific molecular markers, and the advent of RT-qPCR and RQ-PCR have represented a significant advance in the evaluation of MRD over the conventional PCR-based methods[85]. Actually, these methods have been extensively standardized within the EuroMRD Consortium (www.euromrd.org/guidelines, accessed on 27 August 2024), which established guidelines for the analysis and interpretation of the data in order to favor an homogeneous application of MRD studies within different treatment protocols for both pediatric and adult ALL patients.

Regarding the biomarkers used for MRD monitoring, it is known that approximately the 40% of ALL patients have chromosomal translocations (such as *BCR::ABL1*, *ETV6::RUNX1*, *TCF3::PBX1*, *KMT2A::AFF1*). Thus, given that these genetic abnormalities are specific to leukemic cells and remain remarkably stable throughout the disease process, they represent an excellent ”leukemia-specific” target for the assessment of MRD. However, this strategy could be used only in

those leukemia that presents any fusion gene derived from chromosomal translocations[86,87]. In the setting of the Ph-negative ALL, namely those ALL forms which do not present a recurrent chromosomal translocation, the approach must be changed and relies on the use of antigen-receptor gene rearrangements. Specifically, during the maturation of B and T-lymphocytes, the immunoglobulin (IG) and T-cell receptor (TR) genes are assembled through a somatic rearrangement process. Although immunoglobulin and/or T-cell receptor clonal gene rearrangements (IG/TR) are physiological events that are not directly linked to the pathogenesis of leukemia, all leukemic cells will present the same "patient-specific" rearranged clonal IG/TR rearrangement during a neoplastic transformation (Figure 12).

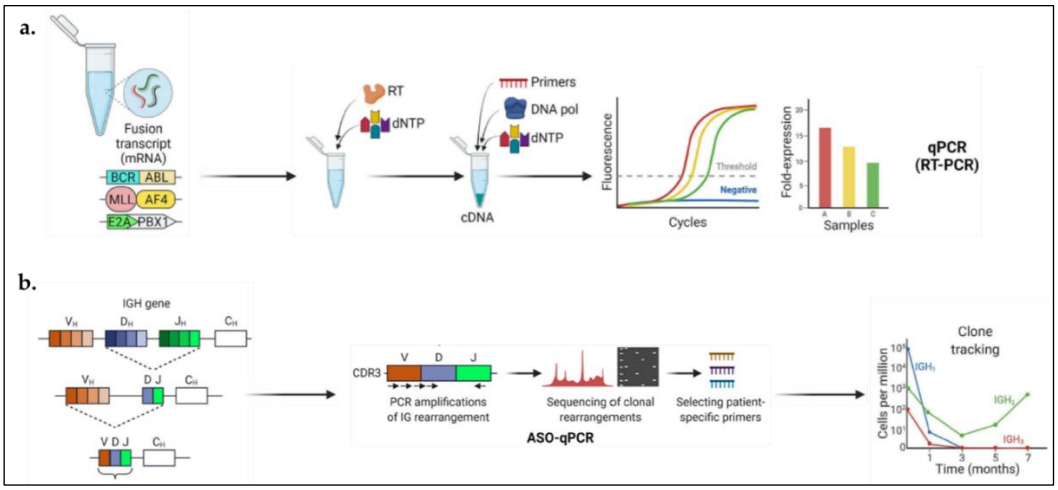


Figure 12 – Molecular methods of MRD detection in ALL: **a.** PCR-based methods for fusion transcripts detection; **b.** workflow for IG/TR clonal rearrangements MRD monitoring (adapted from Saygin C et al, *Hematologica*, 2022).

2.2.1 *BCR::ABL1*-based MRD

The gold standard tool employed for MRD monitoring in Ph+ ALL is quantitative reverse transcription polymerase chain reaction (RT-qPCR). This methodology relies on the in-vitro retro-transcription of mRNA into cDNA, which are then amplified through PCR. The advantages of RT-qPCR include high sensitivity,

speed and low cost and eliminating the necessity for the identification of patient-specific MRD markers. The use of RNA as a substrate for the detection of translocations is more prevalent than that of DNA. This is due to the fact that the precise breakpoint or fusion site of a translocation may occur in different intronic regions and spans extensive segments of DNA, which makes it challenging to determine for each patient. In contrast, the spliced mRNA product (which excludes introns) that gives rise to the fusion transcript is similar between patients and shorter, thus allowing for the use of universal primers. This allows for the application of a single primer set to all patients with the same translocation, facilitating an expedient and straightforward evaluation of fusion transcripts at diagnosis and throughout treatment. Furthermore, these fusion transcripts contribute to the oncogenic process, in contrast to the IG/TR clonal rearrangements. However, RNA is less stable and is a more challenging substrate to work with, given that the transcriptional activity can vary within the blasts and also among different cells within the same leukemic clone, posing a possible hampering in quantitative interpretations of data. The MRD monitoring in Ph+ ALL by the quantitative analysis of the *BCR::ABL1* fusion transcripts have been largely standardized within the EuroMRD consortium that published guidelines for the experimental workflow and data interpretation[88].

In recent years, in the field of *BCR::ABL1*-positive leukemias, i.e. CML and Ph+ ALL, an attempt has been made to overcome the limitations posed by the use of a complicated substrate such as the RNA, both in terms of laboratory management and cell transcriptional activity, through the use of MRD monitoring using genomic DNA (gDNA). The concept relies on the identification of the fusion gene breakpoint through the utilization of either standard (Sanger) or NGS methodologies, thereby enabling the construction of a dedicated TaqMan assay based on the sequence obtained for the subsequently quantitative

monitoring through RQ-PCR. The principal advantage is the development of a patient-specific assay, given the high degree of variability observed in the *BCR::ABL1* fusion gene, which may originate from different breakpoints within the *BCR* and the *ABL1* genes (in some cases, a third gene may also be involved) overcoming the high transcriptional variability typically observed with the RNA[89].

2.2.2 Immunoglobulin/T-cell receptor (IG/TR)-based MRD

Antigen-receptor clonal gene rearrangement analysis is the most widely used method for MRD detection in the ALL setting and has been extensively standardized within the EuroMRD Consortium, which has established guidelines for the analysis and interpretation of RQ-PCR data to promote an homogeneous application of MRD studies[90,91]. Furthermore, IG/TR quantitative MRD monitoring represents the gold standard marker in Ph-negative ALL and in almost all childhood leukemia. The clonal rearrangements of the IG/TR genes are physiological events that are not directly related to the pathogenesis of the leukemia. During the maturation of B and T lymphocytes, the IG and TR genes are reassembled by a somatic rearrangement process. The separate gene segments encoding the V (Variable), D (Diversity) and J (Joining) regions are combined into a single exon encoding the variable region[92]. During this process, some nucleotides are randomly deleted or inserted at the junctions of each segment (N-region), resulting in the final receptor sequences that are unique to each B or T lymphocyte of each individual patient. In the case of neoplastic transformation of a single lymphoid cell, all leukemic cells will present the same rearranged clonal IG and/or TR genes: these combinations can serve as a clonal "fingerprint", allowing each lymphocyte to be identified by its individual recombination. Leukemic blasts are thought to originate from a single B or T cell

clone, making their V(D)J repertoire monoclonal and allowing detection of the patients' specific clonal rearrangements[93,94]. Subsequently, the clonal PCR fragments are sequenced by standard Sanger sequencing (SS), with the objective of defining the junctional regions and obtaining complementary allele-specific oligonucleotide (ASO) primers and TaqMan-probes for MRD monitoring. This process is predominantly performed by RQ-PCR[95]. The amplification conditions and sensitivity testing for each ASO primer are established by a standard curve constructed on the basis of serial dilutions of diagnostic gDNA in a gDNA pool from healthy donors. The standard curve constructed on the diagnostic material is employed to quantify MRD in BM or PB samples collected during and after treatment.

2.2.3 New generation tools for MRD monitoring: digital droplet PCR and next-generation sequencing

Due to the considerable clinical and prognostic value of MRD monitoring, the research in this field is subject to constant evolution. The primary objective is to obtain reliable results and to eliminate potential technical and biological issues. The principal objectives can be broadly categorized into two distinct areas: firstly, to enhance the sensitivity and specificity of the methodologies employed, and secondly, to advance the existing knowledge regarding the most reliable biomarkers, in order to achieve the optimal correlation with disease progression and the clinical course of the patients.

The advent of real-time quantitative PCR technologies (RQ-PCR and RT-qPCR) has signified a substantial advancement in the assessment of MRD in comparison to the conventional PCR-based methodologies. However, this technology presents some intrinsic issues that cannot be entirely resolved. One such issue is the relative quantification of the amplified product. This necessitates the

construction of a calibration curve, which requires enough diagnostic material that, due to clinical or methodological reasons, is not always feasible to store in sufficient quantities. Moreover, a considerable proportion of patients with extremely low MRD levels are currently classified as positive not-quantifiable (PNQ) due to the fact that although the MRD is identified as positive, it is not within the quantitative range when compared with the standard curve of the quantitative RQ-PCR assay. It is therefore necessary to improve the discrimination of these cases, which currently represents a challenge in clinical practice[96,97].

Digital droplet PCR (ddPCR) (Bio-Rad, Hercules, CA) represents the third generation of PCR technology, characterized by the partitioning of the sample into at least 20,000 oil-in-water emulsion droplets. This technique is the most sensitive method for determining the absolute quantification of nucleic acids, both at diagnosis and during treatment in various hematological diseases, without the need for calibration curves[98–100]. ddPCR has been applied in various fields of medical diagnostics, particularly in molecular oncology. Several studies have been conducted to compare the performance of ddPCR with that of quantitative PCR in pediatric and adult patients with a variety of hematological disorders, including Ph+ ALL[101–105]. These studies have established analytical parameters to investigate the applicability of ddPCR for MRD detection and concluded that ddPCR has sensitivity, accuracy and reproducibility at least comparable to that of RQ-PCR and RT-qPCR (Figure 13). Finally, ddPCR is also being used to detect mutations of clinical interest, particularly in the Ph+ ALL setting it has been used to detect a wide range of *BCR::ABL1* KD resistance mutations (i.e. T315I, E255K, V299L, F317L)[106].

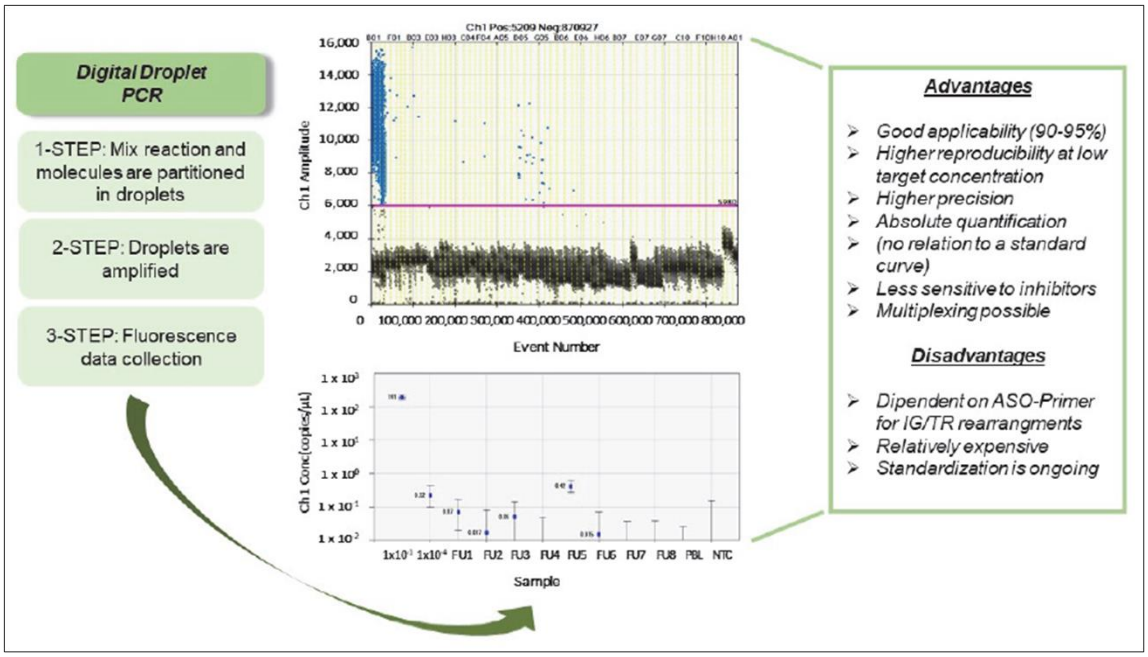


Figure 13 – ddPCR technology: analytical diagram for a ddPCR experiment. (Della Starza I et al, *Cancers (Basel)*, 2023).

NGS technologies have, in general, represented a significant advancement in genomic analysis. Their application can be divided into two main categories: the first is the identification of novel molecular markers that may facilitate an enhanced comprehension of disease biology, and the second is a clinical practical phase for the detection of alterations that could contribute to more effective disease management by improving risk stratification and disease monitoring[107]. This is of particular interest in the field of IG/TR-based MRD monitoring, given that it is not possible to identify a reliable IG/TR marker in approximately 5-10% of cases[108]. This is due to the maturation state of the cell of origin, high immunological clonal variability at diagnosis and technical issues. The integration of NGS technologies into IG/TR marker screening enables the detection and sequencing of any potential IG/TR gene rearrangements with a sensitivity range of 10^{-4} to 10^{-6} [109,110] (Figure 14).

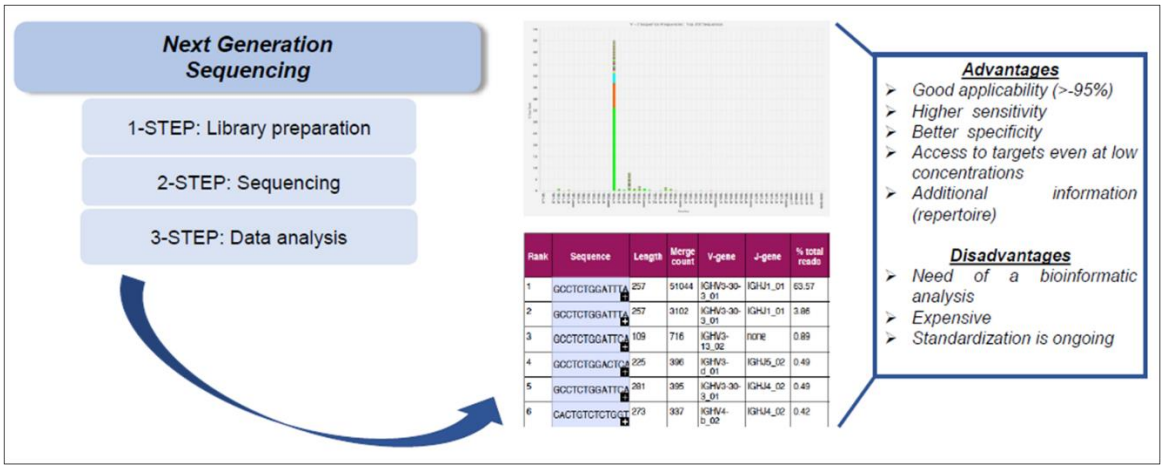


Figure 14 – IG/TR screening by NGS technology. (Della Starza I et al, *Cancers (Basel)*, 2023).

2.3 Typical/lymphoid and CML-like/multilineage Ph+ ALL

In the last years, in Ph+ ALL several groups both in pediatric and in adult settings carried out a formal comparison between IG/TR and *BCR::ABL1* transcript levels evaluation showing in general comparable results in terms of risk of relapse and survival: however, all the studies highlighted that the concordance between these two biomarkers was poor with the *BCR::ABL1* levels persistently positive and significantly higher than IG/TR.

The Czech group, the first to investigate this scenario in the pediatric setting, performed the MRD monitoring using different markers: *BCR::ABL1* fusion transcript, IG/TR and, in order to eliminate drawbacks related to the variability of *BCR::ABL1* mRNA expression, patient-specific gDNA *BCR::ABL1* fusion-breakpoint. The results showed that, while the *BCR::ABL1* MRD data were comparable, the correlation between the *BCR::ABL1* (mRNA and gDNA) levels and IG/TR was poor showing that in at least 20-25% of patients the MRD levels are discordant, with persistent *BCR::ABL1* positivity; moreover, in these patients cell sorting and FISH on diagnostic material assessed the presence of *BCR::ABL1* in various hematopoietic subpopulations (non–ALL B lymphocytes, T-cells,

and/or myeloid cells), defining these patients as "CML-like" due to the possible presence of clonal hematopoiesis resembling a chronic myeloid leukemia scenario[33]. The same group, conducted a retrospective study on 147 Ph+ ALL children, using the gDNA-based monitoring of *BCR::ABL1* fusion-breakpoint and IG/TR confirming that at least the 60% of samples analyzed were discordant with a 25% of patients defined as CML-like (Figure 15). From a clinical standpoint, overall prognosis of CML-like and typical-ALL was similar (5-year-OS 75% and 73%, respectively), though typical-ALL had more relapses while CML-like patients died in the first remission more frequently[111].

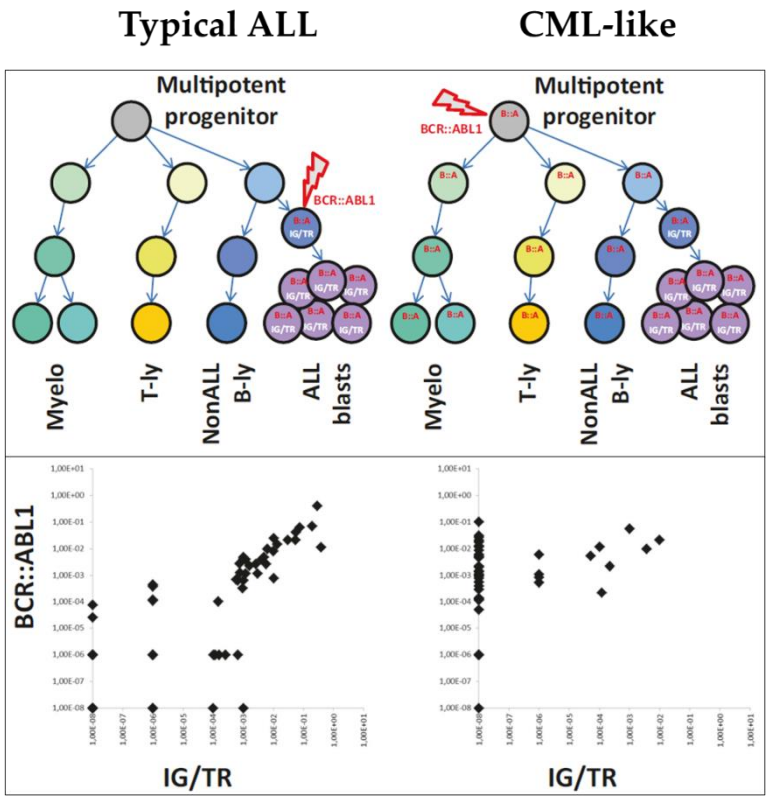


Figure 15 – Schematic illustration of key differences between "Typical ALL" and "CML-like" disease (adapted from Zuna J et al, *Leukemia*, 2022).

Several groups conducted similar comparison analysis, also using different methodological approaches, i.e. NGS, confirming that the concordance rate

between these two biomarkers remains low[112,113], not strictly related to technical or methodologic aspects, but probably sustained by the hypothesis of the presence of a multipotent hematopoietic progenitor that give rise to the spread of the *BCR::ABL1* in a broader cellular types.

More in depth, the French group has recently published the results on the significance of MRD in adult Ph +ALL patients: the authors focused on the use and comparison of these two biomarkers for the identification and characterization of lymphoid and multilineage subgroups. This cohort represents the largest series investigated in the adult setting and analyzed samples derived from 228 patients enrolled in the GRAAPH-2014 clinical trial. The comparison between *BCR::ABL1* and IG/TR MRD revealed discrepancies in 98 (43%) of 228 patients, defined as multilineage Ph+ ALL; of note, all the discordant cases that underwent cell-sorting showed *BCR::ABL1* positivity in other cellular compartments (non–ALL B-lymphocytes, T-cells, and/or myeloid cells) (Figure 16). Moreover, they observed that IG/TR early molecular response was strongly associated with outcome and with the identification of patients who might mostly benefit from allo-HSCT. Finally, the persistence of IG/TR positivity before allo-HSCT was correlated with a high-risk of progression before transplantation, suggesting that the introduction of targeted immunotherapy (i.e. blinatumomab) together with TKIs could be a striking therapeutic approach for MRD control[114].

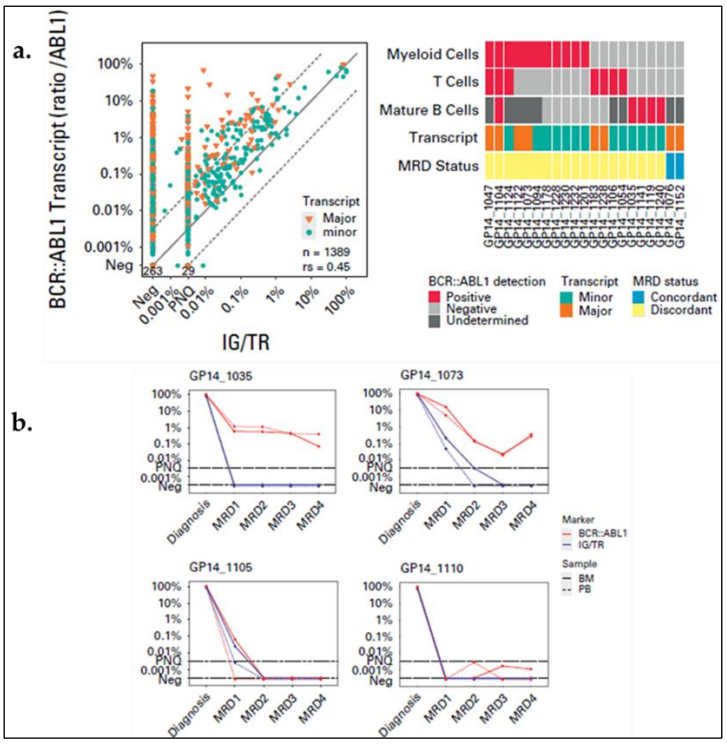


Figure 16 – a. Comparison of MRD levels between IG/TR and *BCR::ABL1* transcripts and heat map of *BCR::ABL1*-positive cell lineage involvements assessed on flow-sorted samples; **b.** Illustrative cases showing discordant (upper panels) and concordant (lower panels) MRD kinetics using *BCR::ABL1* transcripts and IG/TR markers (adapted from Kim R et al, *J Clin Oncol*, 2024)

Regarding molecular features and differences between these two entities, recently two works[115,116] demonstrated how Ph+ ALL is composed by several subclusters based on gene expression profile and genomic alterations. Interestingly, both concluded that multilineage Ph+ ALL is characterized by the deletion of the *HBS1L* gene and the deletion of the chromosome 7, while the lymphoid subcluster is mostly associated with the presence of deletions in the *IKZF1*, *CDKN2A/B* and *PAX5* genes. Overall, from the available literature is possible to conclude that there are at least two subgroups with different developmental trajectories in Ph+ ALL; from a clinical standpoint the impact on survival, relapse or resistance to treatments are scarcely investigated and did not

provide definitive results: this could become extremely relevant especially in the context of the new chemo-free, targeted therapeutic approaches.

3 Refining measurable residual disease monitoring in Philadelphia-positive adult patients: new insights from methodological and molecular standpoints

3.1 Background and aims of the study

MRD negativity represents the primary endpoint in the management of adult Ph+ ALL patients. MRD assays must be highly sensitive and specific. DdPCR and NGS approaches can overcome some limitations of standard methodologies. In order to evaluate the best strategy for MRD monitoring, in this study we performed: i) *BCR::ABL1*-based MRD monitoring between RT-qPCR and ddPCR; ii) MRD evaluation by *BCR::ABL1* fusion transcript and IG/TR clonal gene rearrangements ; iii) a comparison of the MRD concordance rate between the two markers; iii) a correlation with biologic features and clinical outcome.

3.2 Materials and methods

3.2.1 Study population

The present project comprised a total of 167 samples from 111 adults enrolled in the ongoing phase III GIMEMA ALL2820 (NCT04722848) clinical trial. Briefly, the chemo-free experimental arm is based on the sequential administration of ponatinib, a third generation pan-TKI, in the induction phase followed by blinatumomab as consolidation treatment; the control arm is based on a standard chemotherapy backbone together with imatinib[117] (Figure 17).

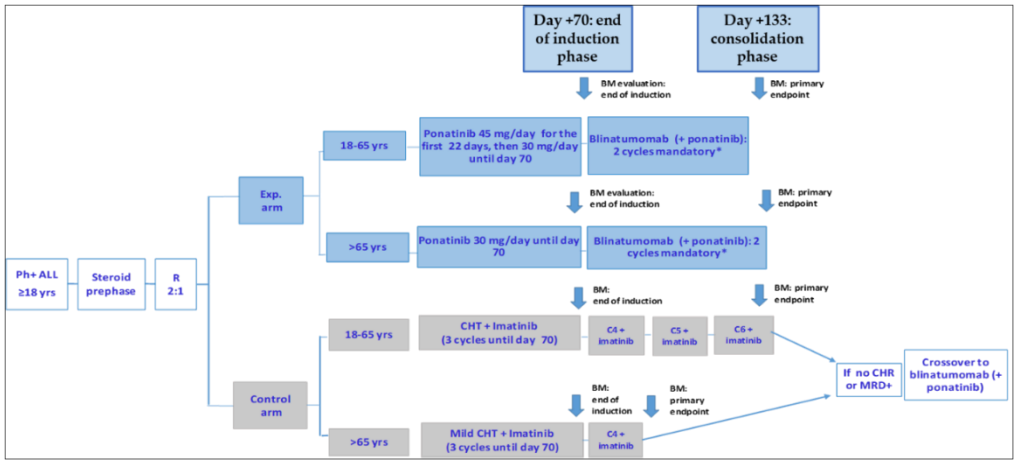


Figure 17 – Schematic illustration of the GIMEMA ALL2820 clinical trial.

Samples derived from patients from both the experimental and the control arm. Established time points were day +70 (end of induction) and day +133 (after 2 cycles of blinatumomab or after at least 4 cycles of chemotherapy, respectively). The baseline characteristics of the whole population are summarized in table 2.

Characteristics	Patients (N = 111)
Age - years	
Median	53
Range	20 - 80
Sex - no. (%)	
Male	63 (56.7)
Female	48 (43.3)
WBC ($x10^9$/L)	
Median	16.4
Range	1.1 - 356
Blast (%)	
Median	68
Range	11 - 97
Fusion protein - no. (%)	
p190	78 (70.3)
p210	27 (24.3)
p190/p210	6 (5.4)
Arm - no. (%)	
Experimental	78 (70.3)
Control	33 (29.7)

Table 2 - Baseline characteristics of the population included in the study.

3.2.2 Nucleic acid extraction (DNA and RNA)

All samples were collected in sodium citrate tubes and maintained at room temperature until further processing. Mononuclear cells, including the lymphocyte component, were isolated from BM and PB samples using a Lymphoprep-based gradient centrifugation.

gDNA was manually extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA); total RNA was manually isolated using Trizol methodology.

After extraction, the gDNA and the RNA were evaluated for concentration and a quality-check through a spectrophotometer by measuring the absorbance to 260 nm, and purity 260/280 absorbances. Finally, both nucleic acids were subjected to an electrophoretic run on agarose gel (2% for gDNA and 1% for RNA).

3.2.3 Reverse transcription

For RNA reverse-transcription and cDNA synthesis, 1 μg of RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen™, CA).

3.2.4 *BCR::ABL1* RT-qPCR

The workflow for RT-qPCR for MRD monitoring by quantification of the *BCR::ABL1* fusion transcript was performed in accordance with the guidelines published by EuroMRD[88]. Briefly, the relative quantification of the transcript requires the use of a plasmid standard DNA of known titer for both the *BCR::ABL1* and the control gene[87]. The universal sequences of primers and TaqMan probes employed for the p190 and p210 isoforms are shown in table 3.

PRIMER	SEQUENCE
BCR::ABL1 p190 FW	5'-CTGGCCCAACGATGGCGA-3'
BCR::ABL1 p210 FW	5'-TCCGCTGACCATCAATAAGGA-3'
BCR::ABL1 RW consensus	5'-CACTCAGACCCTGAGGCTCAA-3'
ABL1 FW	5'-TGGAGATAACACTCTAAGCATAACTAAAGG-3'
ABL1 RW	5'-GATGTAGTTGCTTGGGACCCA-3'
PROBE	**SEQUENCE**
BCR::ABL1 consensus	[FAM] 5'-CCCTTCAAGCGGCCAGTAGCATCTGA-3' [BHQ1]
ABL1	[HEX] 5'-CCATTTTTGGTTTGGGCTTCACACCATT-3' [BHQ1]

Table 3 – Sequences of primers and probes for *BCR::ABL1* fusion transcripts.

3.2.5 IG/TR clonal gene rearrangements screening

Diagnostic gDNA samples were subjected to a standard screening following the EuroMRD guidelines in order to identify rearrangements of the *IGH*, *IGK*, *TRG*, *TRD* and *TRB* genes[118,119]. Briefly, the nucleotide sequences obtained were aligned to the IgBLast database and the international ImMunoGeneTics (IMGT) information system. The status of the clonal immune gene rearrangements was examined and confirmed by homo/heteroduplex analysis. Subsequently, an ASO primer and/or TaqMan probe were designed to be complementary to the junctional regions of the patient-specific marker that had been identified. In cases where standard screening methods failed to identify an IG/TR marker, a NGS approach was employed. This involved the use of the LymphoTrack assay for IGH (FR1/2/3) and IGK (Invivoscribe, San Diego, CA, USA)[120]. Different reports have demonstrated the clonality concordance between the LymphoTrack system and standard molecular screening approaches[121].

3.2.6 Digital droplet PCR MRD monitoring

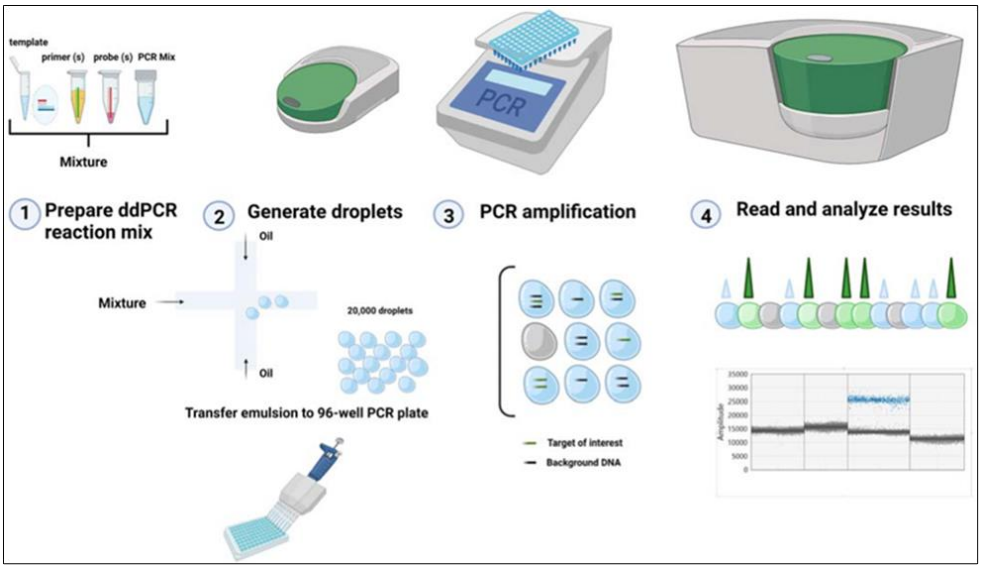


Figure 18 – Schematic overview for a ddPCR experiment (Lambrescu I et al*, Int J Mol Sci,* 2022).

ddPCR for *BCR::ABL1*-based MRD monitoring.

The same *BCR::ABL1* primers and probes used in the RT-qPCR experiments were employed and the manufacturer's protocols were followed. The probes for the targets *BCR::ABL1* p190 and p210 isoforms were labelled with the FAM and BHQ1 reporters, while the probe for the control gene was labelled with the FAM and HEX reporters. The primers and probes were utilized at a final concentration of 900 nmol/L (0.23 µL/well) and 250 nmol/L (0.25 µL/ well), respectively. In each experiment, negative controls were included, namely, samples that were Ph-negative and no-template controls (NTCs). Positive control samples were also employed, comprising diagnostic samples from Ph+ ALL. The *ABL1* gene was employed as a control gene to assess the quality of the material and to calculate the ratio between the target and control gene. For MRD samples 250 ng/well were loaded and tested in triplicate, while for control gene and control samples (positive and negative) 125 ng in order to avoid the saturation of the signal and tested at least in duplicate. The reaction mixture (20 µL), comprising cDNA, 11

μL of ddPCR Supermix for Probes (No dUTP) (Bio-Rad, Hercules, CA), primers and probes, was loaded into the DG8 cartridge wells, covered with the DG8 gasket, and loaded into the QX200 Droplets Generator together with 70 μL of droplet oil, in order to generate the droplets. During the droplet generation process, template molecules are randomly distributed into individual droplets. Subsequently, the droplets were transferred from the DG8 cartridge to a 96-well PCR plate, which was sealed with a pierceable foil heat seal, and amplified through a thermal cycler. The thermal cycling conditions were as follows: one cycle at 95°C for 10 minutes, 40 cycles at 94°C for 30 seconds, 40 cycles at 60°C for one minute, one cycle at 98°C for 10 minutes, and 4°C as the holding temperature. Subsequently, the PCR plate was loaded into the QX200 Droplets Reader and the data were analyzed using the Quantalife QX200 ddPCR software (Bio-Rad Laboratories, CA). In accordance with the manufacturer's instructions, only experiments giving a number of droplets ≥9000/replicate were considered acceptable. The appropriate quantification of each experiment was conducted by establishing a threshold value through manual curation that was sufficiently distant from the background to ensure optimal sensitivity and specificity. Given the random nature of the partitioning, the fluorescence data obtained following amplification can be well fit by a Poisson distribution. This allows the number of template molecules in each droplet to be determined on the basis of the fluorescence data. The absolute concentration was automatically calculated by the analysis software and expressed as copies/μL. Additional technical and analytical parameters of the experiments can be viewed from a previous paper published by our group[122]. For *BCR::ABL1* transcript quantification, the International Scale (IS) was applied, as recommended by the guidelines, using the following ratio: [(*BCR::ABL1*/*ABL1*) x 100].

ddPCR for IG/TR-based MRD monitoring.

First, each designed patient-specific ASO-primer and/or probe assay and common fluorescent probes must be checked by RQ-PCR on the corresponding diagnostic gDNA material serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 5 x 10^{-4}, 10^{-5}) in peripheral blood mononuclear cells (PBMCs) gDNA derived from a pool of healthy donors in order to define the amplification conditions, i.e. best annealing temperature and sensitivity (at least 10^{-4}). All probes for the IG/TR markers were labelled with the FAM and BHQ1 reporters. Subsequently, a primer/probe reaction mixture was prepared, with final concentrations of 1000 pmol and 400 pmol, respectively. In each experiment, negative controls were included, namely gDNA from a pool of healthy PBMCs and NTCs. As positive control, the diagnosis of each individual patient was used, or alternatively, a 10^{-1} dilution was employed. This was done to avoid saturation of the signal, which is necessary for the assessment of the threshold cut-off. A total of 500 ng/well of gDNA was loaded for all samples. MRD samples were run in triplicate, while control samples were run at least in duplicate. The reaction mixture (22 μL), comprising gDNA, 11 μL of ddPCR Supermix for Probes (No dUTP) (Bio-Rad, Hercules, CA) and primer/probe mix, was loaded into the DG8 cartridge wells, covered with the DG8 gasket, and loaded into the QX200 Droplets Generator together with 70 μL of droplet oil, in order to generate the droplets. Next, the droplets were transferred from the DG8 cartridge to a 96-well PCR plate, which was sealed with a pierceable foil heat seal, and amplified through a thermal cycler. The thermal cycling conditions were as follows: 95° C for 10 minutes; 40 cycles at 94° C for 30 seconds; proper Tm° C for 1 minute; 98° C for 10 minutes and 4°C as the holding temperature (the Tm must be adjusted according to each ASO-primers proper annealing temperature). Subsequently, the PCR plate was loaded into the QX200 Droplets Reader and the data were analyzed using the Quantalife QX200 ddPCR

software (Bio-Rad Laboratories, CA). In accordance with the manufacturer's instructions, only experiments giving a number of droplets ≥9000/replicate were considered acceptable. The appropriate quantification of each experiment was conducted by establishing a threshold value through manual curation that was sufficiently distant from the background to ensure optimal sensitivity and specificity. The absolute concentration was automatically calculated by the analysis software and expressed as copies/μL. For the quantification of IG/TR MRD the following ratio was used, in accordance with guidelines[123]: [(N × 20μL) / n] where ***N*** is the mean copies/μL of each merged replicate of each sample; ***20μL*** is the total reaction volume (the value has been conventionally set at 20μL to avoid any potential bias). ***n*** is defined as the total number of cells of the gDNA used (500 ng), corresponding to approximately 75000 cells.

In both MRD experiments, the interpretation of the ddPCR data was carried out as follows:

- **Positive sample**: a merge of events ≥ 3, regardless of number of positive replicates;
- **PNQ sample**: a merge of event = 2;
- **Negative sample**: a merge of event ≤ 1.

A sample was considered as discordant when the MRD values assessed with the two distinct markers did not give a concordant result.

3.2.7 Multiplex Ligation-dependent Probe Amplification (MLPA)

A comprehensive MLPA analysis was conducted on all patients at the time of diagnosis. This analysis aimed to identify CNVs in a panel of clinically significant genes, including *IKZF1*, *CDKN2A/B*, *EBF1*, *BTG1*, *ETV6*, *RB1*, and *CRLF2*.

Specifically, it was employed for the screening of the *IKZF1*^{plus} signature. gDNA extracted from BM or PB samples with at least 30% blasts infiltration were denatured and hybridized using the SALSA probemix P335-C2 ALL-IKZF1 (MRC Holland, Amsterdam, NL). A fragment analysis was conducted using a 3500 Genetic Analyzer sequencer (Applied Biosystems, Life Technologies) and the resulting data were analyzed using the Coffalyser.Net software (MRC Holland). The interpretation of the results was performed in accordance with the manufacturer's guidelines (Figure 19).

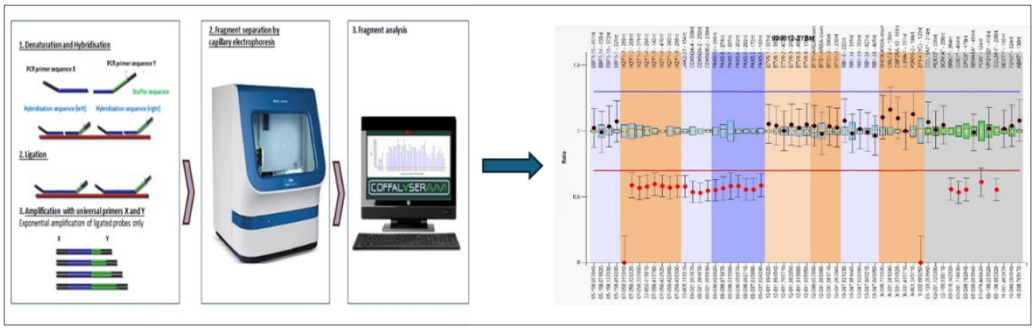


Figure 19 – Schematic overview of a MLPA experiment.

3.3 Results

3.3.1 *BCR::ABL1*-based MRD comparison between RT-qPCR and ddPCR

Firstly, in order to confirm the reliability and applicability of ddPCR in MRD monitoring, a comparative study was conducted on 156 samples (on the basis of available material) between the gold standard methodology, i.e. RT-qPCR, and ddPCR using the *BCR::ABL1* transcript as marker. Overall, *BCR::ABL1*-based MRD comparison showed a correlation degree of 0.93 (R^2) with discordances falling mostly in cases with low MRD levels (Figure 20).

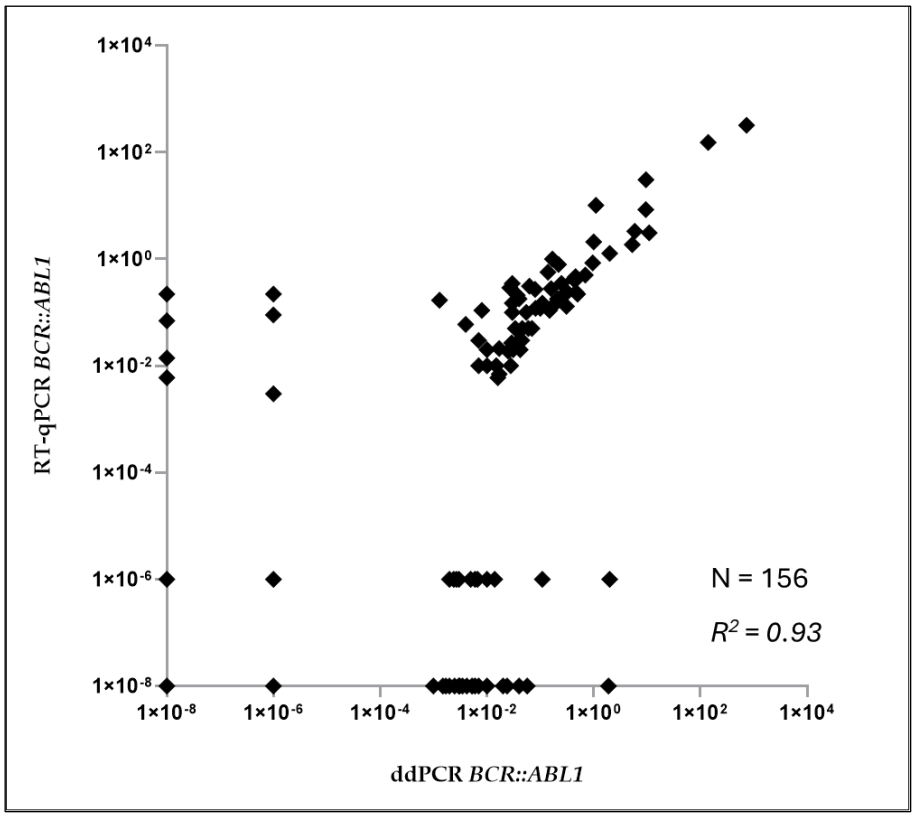
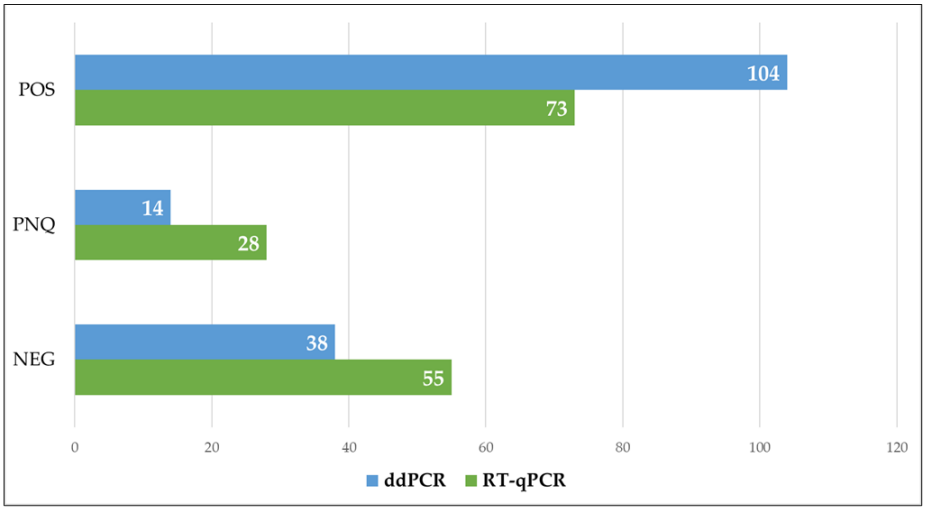


Figure 20 – *BCR::ABL1*-based MRD comparison between RT-qPCR and ddPCR.

Among RT-qPCR positive cases, a concordance of 90.4% was observed, with 66/73 samples positive also by ddPCR; of note, within the 28 PNQ samples by RT-qPCR 13 (46.4%) were positive and quantifiable, 11 (39.3%) were negative and only 4 (14.3%) remained PNQ. Finally, within 55 negative cases by RT-qPCR, 23 (41.8%) were confirmed negative, while 7 (12.7%) became PNQ and 25 (45.5%) became positive and quantifiable. Results are summarized in figure 21. Thus, the overall concordance rate between RT-qPCR and ddPRC *BCR::ABL1*-based MRD monitoring was 59.6%.

a.



b.

N = 156	RT-qPCR POS	RT-qPCR PNQ	RT-qPCR NEG
ddPCR POS	66	13	25
ddPCR PNQ	3	4	7
ddPCR NEG	4	11	23

Figure 21 – a. Overall *BCR::ABL1*-based MRD results; **b.** *BCR::ABL1*-based MRD concordance (in red are the concordant cases).

3.3.2 Copy number variations

All diagnostic samples from the 111 patients were screened by MLPA for CNVs with a panel of genes of clinical and biological interest in the context of Ph+ ALL, with the specific aim of identifying the *IKZF1*^{plus} profile. Overall, regarding the *IKZF1* gene, 39/111 (35.1%) patients were wt, 39/111 (35.1%) exhibited loss of *IKZF1* only, and 33/111 (29.8%) were *IKZF1*^{plus}. With respect to the other genes included in the panel: the most frequently deleted gene was *CDKN2A/B* followed by *PAX5* with 36/111 (32.4%) and 33/111 (29.8%) patients presenting deletions, respectively; as expected, given that deletions in these genes contribute to the definition of the *IKZF1*^{plus} signature. Conversely, gain in *CRLF2* was observed in

10/111 (9%) patients, which represented the gene most affected by gain (Figure 22).

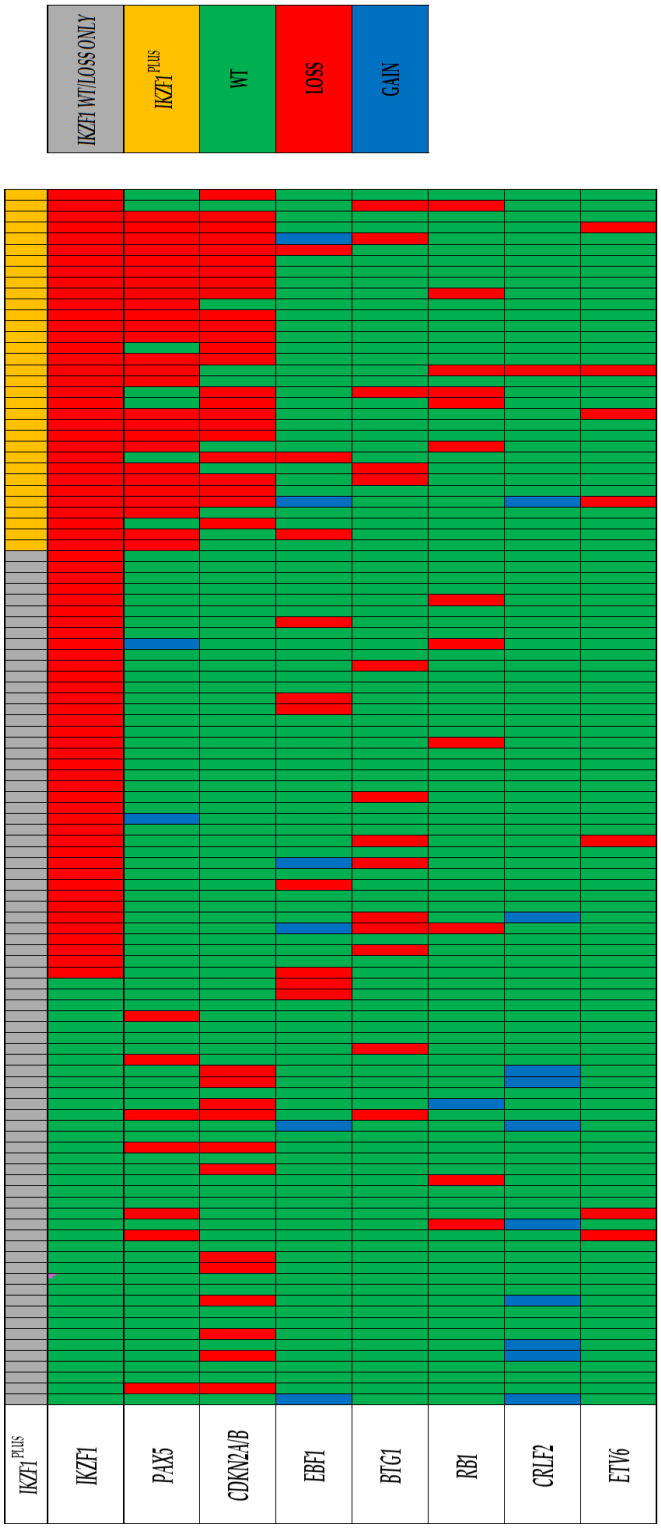


Figure 22 – Heatmap of the CNV at baseline of the whole cohort.

3.3.3 Double hit strategy for MRD monitoring using *BCR::ABL1* and IG/TR as markers

At diagnosis, all the 111 patients were screened for a clonal IG/TR rearrangement to be used as MRD marker. A clonal IG/TR rearrangement was identified in 105/111 (94.6%) cases; however, in 8 (8.2%) it was not possible to obtain a sufficiently reliable ASO-primer for MRD monitoring because the sensitivity was below 10^{-4} during preliminary tests. Finally, 6/105 (5.4%) patients lacked a clonal IG/TR rearrangement. Overall, 97/111 (87.4%) patients were evaluable for IG/TR MRD monitoring (Figure 23).

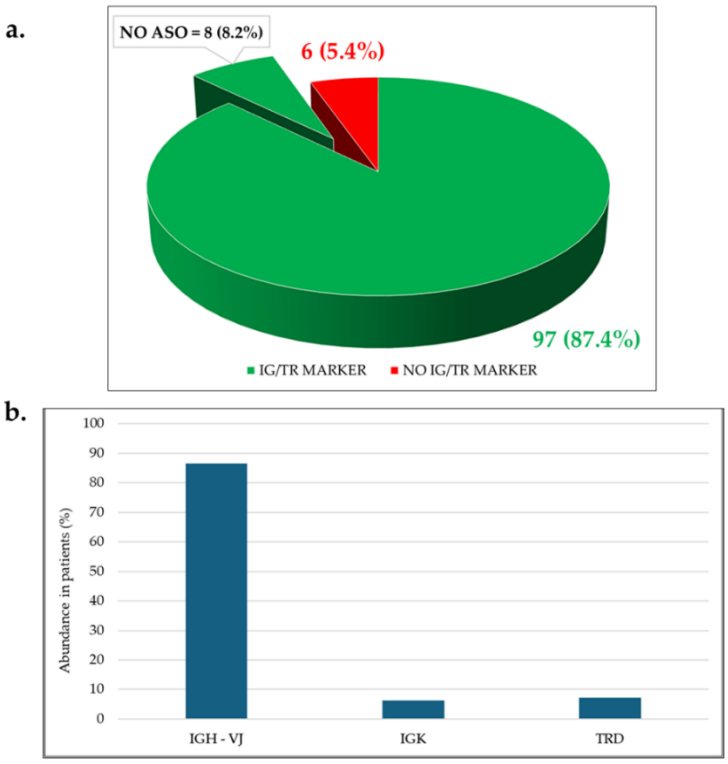
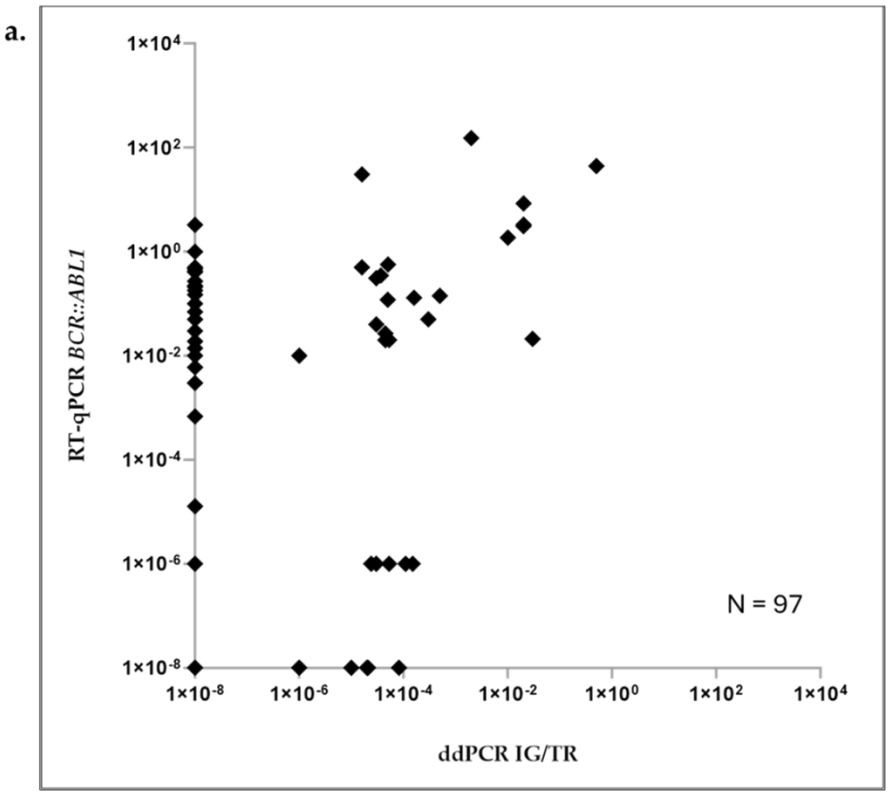


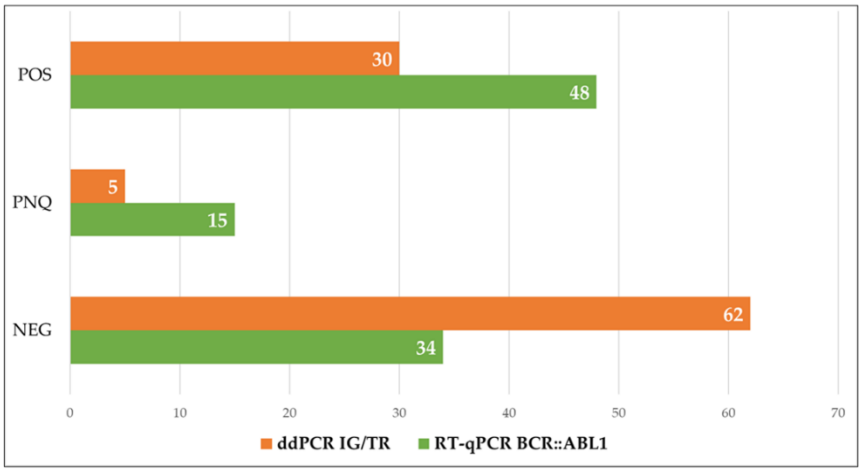
Figure 23 – IG/TR clonal rearrangements screening at baseline: **a.** overall results**; b.** abundance of IG/TR clonal rearrangements type.

At day +70, the overall concordance rate between *BCR::ABL1* and IG/TR was 46.4%; 48/97 (49.5%) cases were *BCR::ABL1*^{pos}; 20 were concordantly IG/TR^{pos}

(41.7%), 1 was IG/TR^{PNQ} and 27 were IG/TR^{neg}; 34/97 (35%) cases were *BCR::ABL1*^{neg}, 25 of which were concordantly IG/TR^{neg} (73.5%), 5 were IG/TR^{pos} and 4 were IG/TR^{PNQ}; finally, 15/97 (15.5%) cases were *BCR::ABL1*^{PNQ}, 5 of which were IG/TR^{pos} and 10 were IG/TR^{neg} (Figure 24).



b.



c.

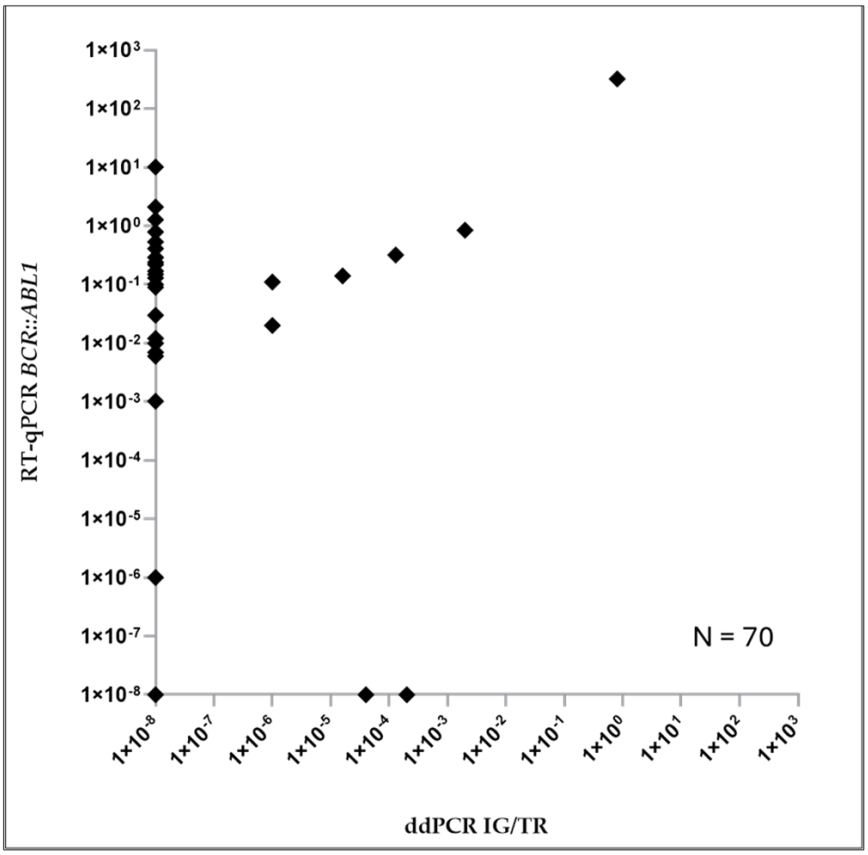
N = 97	ddPCR IG/TR POS	ddPCR IG/TR PNQ	ddPCR IG/TR NEG
RT-qPCR *BCR::ABL* 1 POS	20	1	27
RT-qPCR *BCR::ABL1* PNQ	5	/	10
RT-qPCR *BCR::ABL1* NEG	5	4	25

Figure 24 – Day +70: **a.** MRD comparison between *BCR::ABL1* and IG/TR; **b.** overall BCR::ABL1 and IG/TR MRD results; **c.** *BCR::ABL1* and IG/TR MRD concordance (in red are the concordant cases).

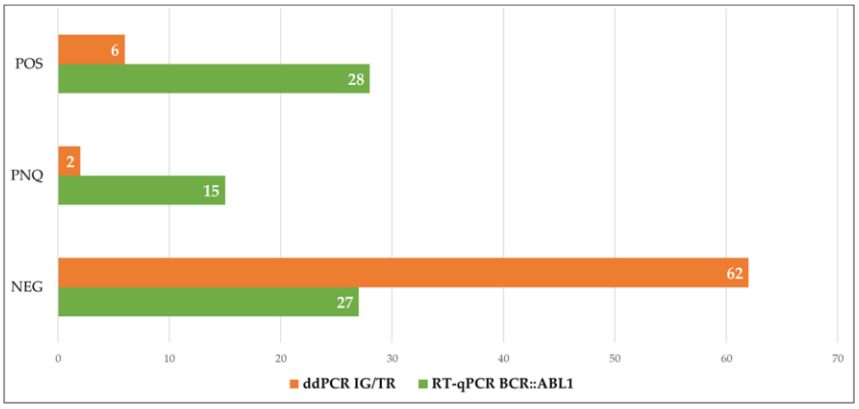
The concordance rate was similar between *IKZF1*^{plus} vs *IKZF1* wt/*IKZF1* loss (48.4% vs 44%), p190 vs p210-p190/p210 (47% vs 42%) cases and between experimental arm (43.4%) and control arm (51.6%).

At day +133, 70 patients were studied and the overall concordance rate was 41.4%: 28/70 (40%) cases were *BCR::ABL1*^{pos}, 4 of which were concordantly IG/TR^{pos} (14.3%), 2 were IG/TR^{PNQ} and 22 were IG/TR^{neg}; 27/70 (38.6%) cases were *BCR::ABL1*^{neg}, 25 of which were concordantly IG/TR^{neg} (92.6%) and 2 were IG/TR^{pos}; finally, 15/70 (21.4%) cases were *BCR::ABL1*^{PNQ} and IG/TR^{neg} (Figure 25).

a.



b.



c.

N = 70	ddPCR IG/TR POS	ddPCR IG/TR PNQ	ddPCR IG/TR NEG
RT-qPCR *BCR::ABL1* POS	4	2	22
RT-qPCR *BCR::ABL1* PNQ	/	/	15
RT-qPCR *BCR::ABL1* NEG	2	/	25

Figure 25 – Day +133: **a.** MRD comparison between *BCR::ABL1* and IG/TR; **b.** overall *BCR::ABL1* and IG/TR MRD results; **c.** *BCR::ABL1* and IG/TR MRD concordance (in red are the concordant cases).

A significative lower concordance was observed in the *IKZF1*^{plus} (24%) vs *IKZF1* wt/*IKZF1* loss (51%) (p = .042), while no significant concordance differences were observed in the p210-p190/p210 (27.3%) compared to p190 (48%) cases, although a lower concordance is evident in the p210-p190/p210 group, and between experimental arm (41.6%) and control arm (40.9%). Five patients experienced a hematologic relapse. Dual monitoring was feasible in 4/5 cases (in 1 material was lacking) and, in addition to the evaluation at day +70 and +133, a retrospective backtracking was carried out at a previous time-point: 1 was concordantly *BCR::ABL1*^{pos} (*BCR::ABL1*/*ABL1* x 100 = 0.26) and IG/TR^{pos} (4.0E^{-04}), 1 was *BCR::ABL1*^{pos} (*BCR::ABL1*/*ABL1* x 100 = 7.12) and IG/TR^{neg}, while the other 2 cases were both *BCR::ABL1*^{neg} but IG/TR^{pos} at 4.0E^{-05} and 2.0E^{-04}, respectively, suggesting the presence at the onset of non Ph+ subclone.

Overall, for the 70 patients who could be monitored with both markers at both TPs, with regard to MRD monitoring by *BCR::ABL1*, 26/70 (37.1%) patients had reached the CMR at day +70: 17 were confirmed negative, 5 became PNQ and 4 reverted to a positive status; 33/70 (47.1%) patients had not reached the CMR at day +70: 21 were confirmed positive, 8 became PNQ and 4 became negative at day +133. Finally, 11/70 (15.8%) patients were PNQ at day +70: 2 confirmed PNQ, 6 became negative and 3 became positive (Figure 26).

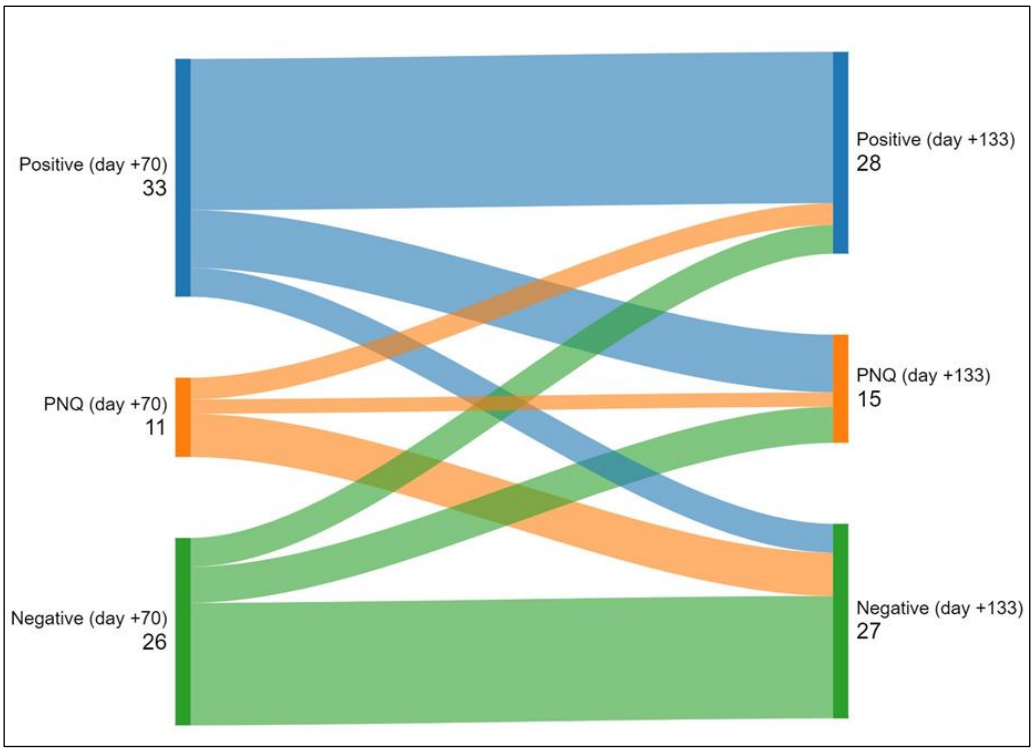


Figure 26 – *BCR::ABL1* MRD by RT-qPCR maintenance status between day +70 and day +133.

Regarding to MRD monitoring by IG/TR, 47/70 (67.1%) patients had reached the CMR at day +70: 43 were confirmed negative, 1 became PNQ and 3 reverted to a positive status; 20/70 (28.6%) patients had not reached the CMR at day +70: 3 were confirmed positive, 1 became PNQ and 16 became negative at day +133. Finally, 3/70 (4.3%) patients were PNQ at day +70 and became negative (Figure 27).

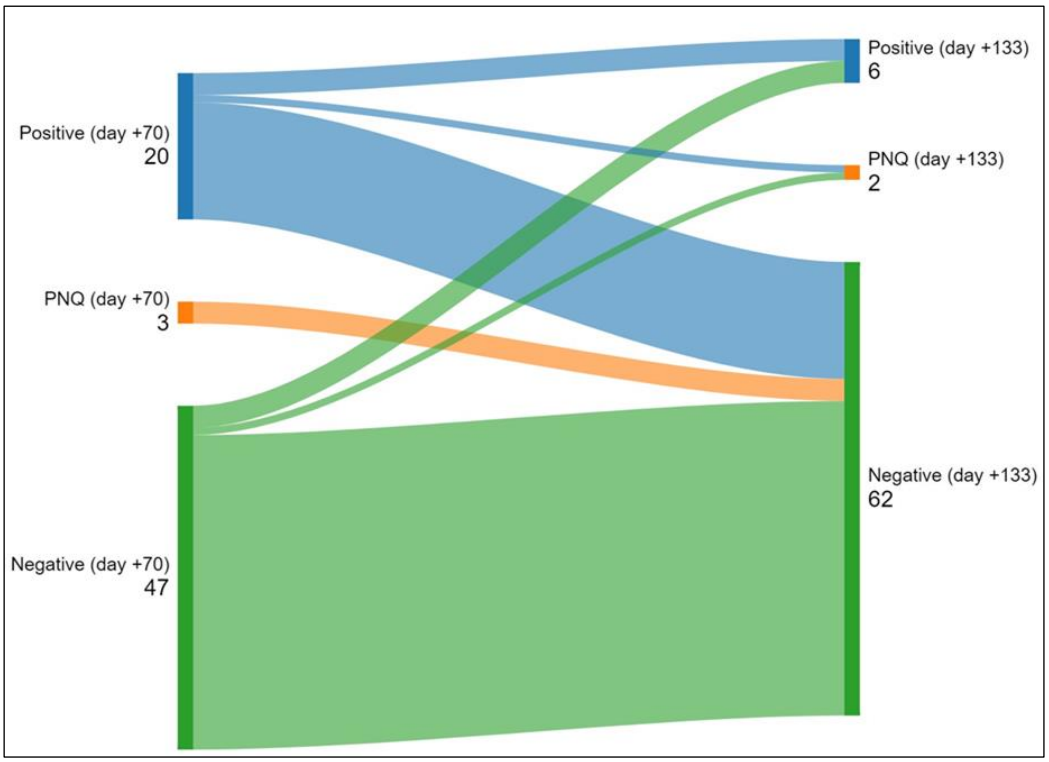


Figure 27 – IG/TR MRD by ddPCR maintenance status between day +70 and day +133.

3.3.4 Case study: refractory Philadelphia-positive acute lymphoblastic leukemia carrying an *IKZF1*^{plus} profile

Here, we report a case of a Ph+ ALL patient with an *IKZF1*^{plus} profile enrolled in the experimental arm of the GIMEMA ALL2820 clinical trial who experienced an extremely aggressive disease that prompted us to perform an in-depth molecular examination, in order to understand possible resistance mechanisms. This patient could not be included in the main cohort due to the lack of sufficient biological material at the primary endpoints of the present study.

A 49-year-old male with marked hyperleukocytosis (217.4 x 10^9/L) was admitted to the hematology unit of of Istituto Tumori "Giovanni Paolo II" in Bari in November 2021. Immunophenotypic characterization revealed the presence of 92% of B-common lymphoblasts in the PB and molecular analysis showed a p190 *BCR::ABL1* fusion protein; results were confirmed also in BM aspirate. Being a

Ph+ ALL, he was enrolled in the phase-3 GIMEMA ALL2820 frontline trial and randomized in the experimental arm. At diagnosis, CNVs analysis by MLPA showed genic deletions in the *IKZF1* (exons: 1, 4-7), *CDKN2A* (exons: 2,4) and *CDKN2B* (exon 2) genes thus fulfilling the definition of an *IKZF1*^{plus} signature. The patient started, as per protocol guidelines, induction with ponatinib at 45 mg/daily together with steroids and intrathecal central nervous system prophylaxis. The dose of ponatinib was reduced to 15 mg/daily due to liver and cardiovascular toxicity and, at resolution increased to 30 mg. End of induction, i.e. day +70 from treatment start, assessment showed a complete hematologic remission whereas real-time PCR showed MRD (*BCR::ABL1*/*ABL1* x 100 = 0.24) persistence. SS demonstrated the occurrence of a T315I *ABL1* mutation, confirmed by ddPCR. The presence of the mutation already at diagnosis and/or at previous time-points was excluded by ddPCR analysis; *ABL1* compound mutations and *TP53* alterations were also ruled out by SS. Since the patient was nevertheless in hematologic remission, as per protocol guidelines, consolidation with blinatumomab was administered and a MRD improvement was observed after cycle 1 (*BCR::ABL1*/*ABL1* x 100 = 0.01). Unfortunately, a full-blown hematologic relapse occurred after the 2nd blinatumomab cycle. Salvage therapy with inotuzumab ozogamycin induced a complete molecular response, that enabled to carry out an allo-HSCT from a matched sibling donor following a myeloablative conditioning, (no complications documented). Unfortunately, a second hematologic relapse with a marked hyperleukocytosis (71.4 x 10^9/L) occurred however 38 days after allo-HSCT. The patient was therefore eligible for anti-CD19 CAR-T cell salvage therapy, but lymphapheresis proved successful only at the third attempt. Given the advanced chemorefractory disease, the association of ponatinib and venetoclax was used as bridging-therapy during the

CAR-T cell turn-around time, but the patient eventually died due to disease progression and infectious complications.

In order to understand the biological factors contributing to such an aggressive scenario, several assays were used to screen this case. First, since at diagnosis the conventional cytogenetics failed, we could infer the patient's karyotype by d-MLPA analysis (SALSA® digitalMLPA™ Probemix D007 Acute Lymphoblastic Leukemia, MRC Holland, Amsterdam, NL)[124,125] that showed several chromosomal alterations: heterozygous deletions at 7p12.2 involving the *IKZF1* gene and at 9p21.3 involving the *CDKN2A/B* genes; homozygous deletions at 9p21.3 involving the *MTAP* and *CDKN2A* genes and at 14q32.33 involving the *IGHM* gene; genic duplications within the Xp(PAR) region at Xp22.33 involving several genes including *CRLF2* (Figure 28).

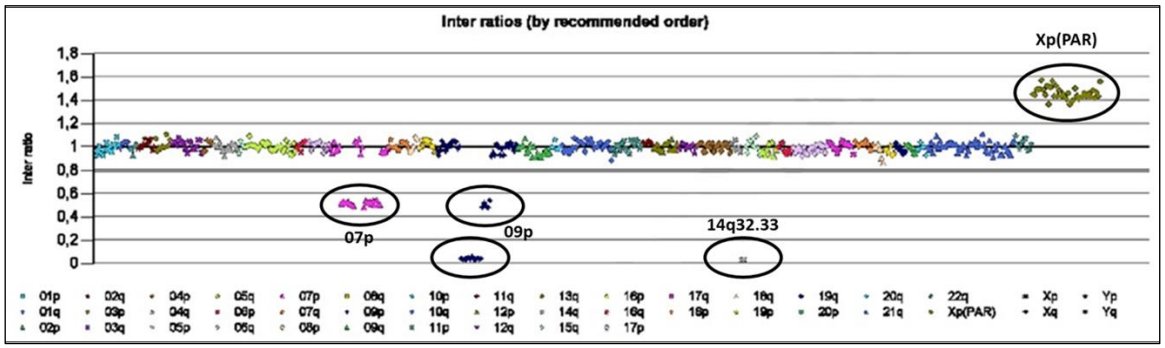


Figure 28 – d-MLPA analysis on diagnostic sample.

Moreover, based on a previous work from our group that demonstrated a significative correlation in Ph+ ALL adult patients between the presence of two different deletions in the *MEF2C* gene and a better outcome in terms of achievement of CMR and better DFS compared to the wild-type cases, we tested the diagnostic sample by ddPCR using a custom assay for *MEF2C* CNV which proved negative. At diagnosis, the clonal IG/TR marker screening showed an

aberrant T-cell receptor clonal gene rearrangement (*TRGV3*01-TRGJ1*02*) that was used as marker for MRD evaluation by a patient-specific Taq-Man assay (Table 4).

PRIMER	SEQUENCE
PFW	5'-ATCTAATTGAAAATGATTCTGGGGTCTA-3'
PRW	5'-AAGGTAATAGAGGGAAAGCAGGAAAT-3'
PROBE	[FAM] 5'-CTGGGACAGGCAGAATATTATAAGAAACTC-3' [BHQ1]

Table 4 – Sequences of patient-specific primers and probe designed on the *TRGV3*01-TRGJ1*02* IG/TR clonal rearrangement.

First, we confirmed the presence of the same leukemic clone at both relapses by SS and then we tested the presence of MRD by ddPCR, following the EuroMRD guidelines for data interpretation, at the time-point prior to the first relapse, (i.e. after the 1st blinatumomab cycle, *BCR::ABL1*/*ABL1* x 100 = 0.01), observing a positivity of $1E^{-04}$ already at this earlier time-point, concordant with the standard *BCR::ABL1* MRD monitoring.

Whole-exome sequencing (WES) was performed on diagnostic, 1st relapse and germinal DNA (extracted from saliva collection) to investigate the presence of additional genetic alterations[126]. Interestingly, WES revealed a pathogenic stop-gain mutation R352* (c.1054C>T; rs746165168) on the *SDHA* gene on diagnostic and 1st relapse sample with a Variant Allele Frequency (VAF) of 41% and 18%, respectively; moreover, on diagnostic sample we observed a missense mutation of uncertain significance S424P (c.1270T>C; rs2056451534) on the *RUNX1* gene with a VAF of 29%. Of note, both mutations were absent at germinal level. Finally, we focused on the *SDHA* R352* pathogenic mutation and in order to validate its presence also at the RNA level, we first performed targeted RNA-sequencing using the TruSight RNA Pan-Cancer Panel Kit (Illumina, San Diego, CA)[43] on

diagnostic and 1st relapse samples that confirmed the presence of the mutation and in order to check for the presence of this mutation also on the 2nd relapse sample at RNA level we performed SS on diagnostic, 1st and 2nd relapse samples that confirmed the results of the WES and showed the persistence of the mutation also at the time of the 2nd relapse (Figure 29)

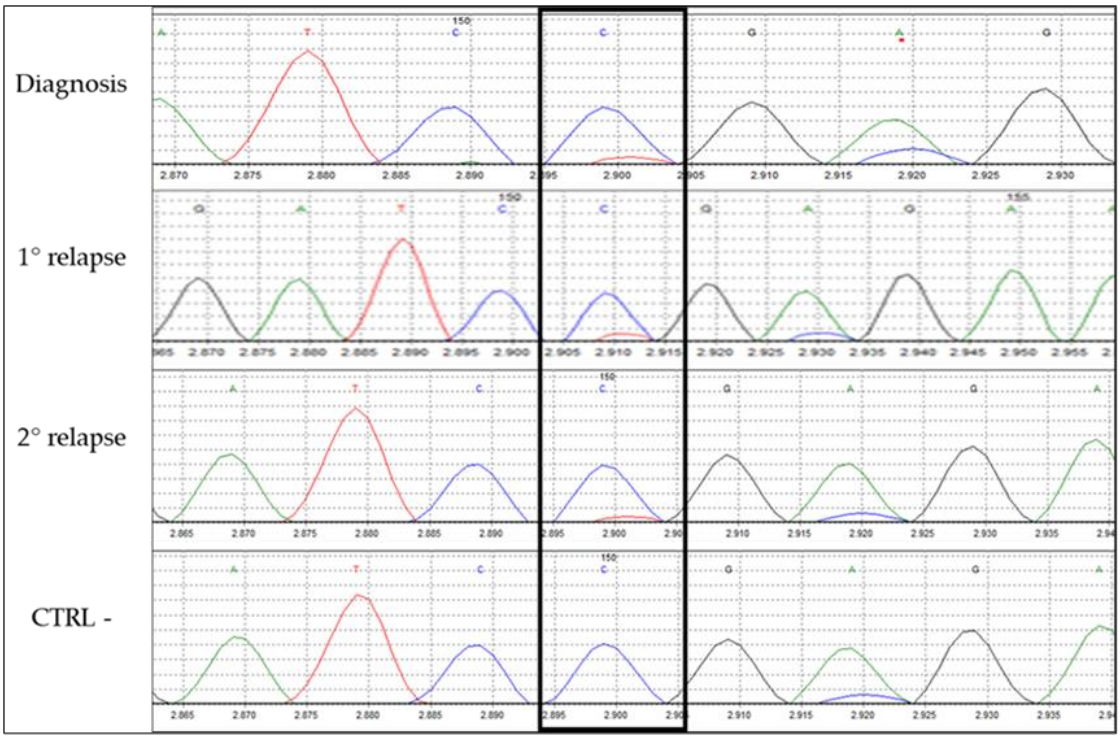


Figure 29 – SS for *SDHA* R352* (c.1054C>T) mutation identification.

4 Discussion and conclusions

Until recently, Ph+ ALL was considered to be the lymphoid leukemia with the poorest prognosis and, in general, one of the most fatal diseases in the field of medicine. Allo-HSCT was regarded as the sole potential therapeutic strategy, although it was only applicable to a limited number of patients. The situation underwent a dramatic transformation with the advent of the first TKI, imatinib, into clinical practice. In general, the introduction of the target therapy, specifically designed to inhibit the uncontrolled hyperactivation of the Philadelphia chromosome-derived fusion protein, the *BCR::ABL1*, which is the driving lesion of the disease, had a revolutionary impact on the care of patients with Ph+ ALL. A further advance in the clinical practice was the introduction of new classes of drugs in the consolidation phase, which were able to replace chemotherapy. This effectively allowed the transition towards a chemo-free therapeutic approach. MoAb, and more specifically, blinatumomab, have demonstrated considerable efficacy, yielding particularly promising outcomes in several clinical trials. This is exemplified by the GIMEMA LAL2116 phase 2 clinical trial, where the combination therapy involving blinatumomab and the second-generation TKI dasatinib during induction led to an OS and DFS rate exceeding 80%. In parallel with the advances in clinical and therapeutic practice, research has also made significant progress. This has been particularly evident in the biological aspects involved in the pathogenesis of the ALL, where new mutations, lesions and chromosomal aberrations have been identified. In the field of translational research, the aim has been to directly apply new knowledge and technologies to support the clinic. This has involved developing tools and guidance to support clinicians in choosing suitable treatment schemes and increasingly accurate prognostic indications. Specific focus was directed towards

the MRD analysis, given that ALL represents the first neoplasm for which the assessment of early response to therapy by MRD monitoring has been demonstrated to be a pivotal tool for guiding therapeutic choices and an independent prognostic factor.

In the field of adult Ph+ ALL, particularly regarding the MRD monitoring, there have been significant developments that have impacted both the implementation and development of novel, increasingly sensitive and specific methodologies for the diagnosis and monitoring of the disease and its response to treatment. The ddPCR represented a significant advancement compared to RT-qPCR technology, offering enhanced sensitivity for detecting specific targets. In multiple comparative analyses, it was found to be at least comparable with the established gold standard method. Its ability to detect low disease rates enabled the minimization of the cases defined as PNQ, which would otherwise be classified as non-quantifiable due to their disease levels falling below the quantifiable range (based on EuroMRD guidelines). This has been identified as a challenging area in the interpretation of data and its utilization in patient management. NGS methods have been a foundational tool in facilitating further research into genetics and molecular processes, especially in the onco-hematologic field. Regarding MRD monitoring in Ph+ ALL, NGS has recently emerged as a pivotal approach, driven by studies that have enhanced the utilization of a second biological marker, namely the clonal rearrangements of IG/TR, in conjunction with the established marker represented by the *BCR::ABL1* fusion transcript. The clonal rearrangements of IG/TR represent the standard marker in the setting of pediatric and adult Ph-negative ALL. However, recent studies have yielded intriguing findings, as it has been observed that in Ph+ ALL the IG/TR clonal rearrangements, despite being mostly discordant with the levels of *BCR::ABL1*, may possess a notable prognostic role with respect to the risk of

relapse, especially post allo-HSCT. The subsequent studies that employed the "double-hit" strategy and monitored MRD levels using these two markers reached the conclusion that Ph+ ALL is not a single disease entity, but can be divided into two distinct subgroups: Ph+ ALL typical/lymphoid variant characterized by the presence of the *BCR::ABL1* only in the leukemic B-cells and the Ph+ ALL CML-like/multilineage variant that exhibits the *BCR::ABL1* expression not only in leukemic blasts but also in other healthy cell types, including non-ALL B-cells, T-cells and myeloid cells, resembling a clinical scenario similar to CML. Based on this data, it was suggested a loss of reliability of the *BCR::ABL1* transcript as marker for monitoring MRD, as it is no longer unique and representative only of neoplastic cells. The observations and hypotheses derived from comparisons of MRD levels between the two markers were subsequently validated by cell sorting studies, which enabled the separation of the various cell populations and their subsequent investigation to detect the presence of *BCR::ABL1*. This led to the confirmation of the existence of these two entities, which are now included in the official classifications.

The present study comprises an in-depth comparative analysis of MRD monitoring from both a methodological and a biological standpoint. The analysis is based on a cohort of 111 adult patients with Ph+ ALL. In addition, the case of a patient who could not be included in the main cohort due to a lack of biological material at the predetermined TP is described as a case report. Firstly, a comparison was made between the gold standard method MRD monitoring, i.e. RT-qPCR, and ddPCR using the *BCR::ABL1* fusion transcript as marker. The observed correlation between the two methods was high (R^2=0.93), with a concordance of 59.6%. In detail, positive samples in RT-qPCR were also substantially confirmed by ddPCR in 90.4% of cases. Conversely, as expected, the major differences were obtained in the PNQ and negative samples by RT-qPCR,

confirmed only in 14.3% and 41.8% of cases, respectively. The results confirm the reliability of ddPCR in detecting samples with a high level of MRD and, simultaneously, its capacity to identify low rates of disease. In fact, the number of PNQ cases has been reduced allowing the redefinition of samples as positive or negative in 46.4% and 39.3% of cases, respectively. These data confirm the findings of a previous study conducted by our laboratory, in which the same correlation was established on a different cohort comprising diagnostic and MRD samples. Similarly, Ansuinelli et al.[122] also identified a high concordance between diagnostic and highly positive MRD samples, while only 24.9% of cases were confirmed in ddPCR, enabling the redefinition of disease levels in the remaining samples. At diagnosis, an IG/TR marker was identified in 94.6% of patients, in line with literature. Furthermore, 97/111 (87.4%) patients were monitored using this marker. MRD monitoring was conducted at the two primary endpoints of the GIMEMA2820 ALL clinical trial, namely the end of the induction phase (day +70) and during the consolidation phase (day +133). The overall concordance rate was 43.9%. No significant differences were identified between the different subgroups analyzed, with the exception of a significantly reduced concordance rate at day +133 between patients presenting the *IKZF1*^{plus} signature and those *IKZF1* wt/loss only, 24% and 51% respectively (*P*=.042). The observed concordance rate of MRD levels appears to be within the expected range, given the existing literature, since the majority of primary studies have documented a concordance rate between 20% and 60%[89,111,113,114]. Five morphological relapses were observed in the study, and in 4/5 cases MRD levels could be retrospectively observed at the TP prior to the overt relapse. Interestingly, in 2/4 cases the samples were found to be *BCR::ABL1*^{neg} but IG/TR^{pos} at the pre-relapse TP with MRD levels of 4.0E^{-05} and 2.0E^{-04}, respectively. Although the levels of residual disease detected were relatively low, it is surprising that *BCR::ABL1* was not

detected, suggesting that in these patients, the ability to monitor MRD with a second marker, i.e. IG/TR, would have allowed for targeted patient management and possibly anticipation of disease recurrence. In addition, the simultaneous *BCR::ABL1* negativity and IG/TR positivity alone could indicate the presence of a second leukemic subclone already present at diagnosis, Ph-negative, which may have been selected during therapy and gained a proliferative advantage, while the Ph+ clone was controlled by the TKI. This situation, in which there is a persistent positivity of IG/TR with respect to *BCR::ABL1*, appears to contrast with the definition of Ph+ ALL multilineage/CML-like, in which the opposite is generally true. It could represent an even different scenario, in which some patients at diagnosis present different leukemic clones, predominantly Ph+, as *BCR::ABL1* can guide the diagnostic work-up. However, over the clinical course, these subclones may be selected to drive disease relapses, which would represent a different pathology from the initial one. In such cases, changes to the therapeutic scheme may be required. In order to confirm this hypothesis, it would be useful to study the diagnostic sample using methods such as RNA-sequencing to investigate the potential presence of additional fusion genes in addition to *BCR::ABL1*. At this time, it is not feasible to establish clinical correlations due to the limited follow-up period and the necessity for an expanded patient cohort. Additionally, given the extremely low relapses and adverse event rates observed, assertions regarding the relative reliability of the two markers in providing prognostic information are premature. Moreover, although the presence of one or two discordant MRD points is an important indication, further study on the individual patients using methods that allow the separation of different cell populations is necessary to establish with certainty how many of the discordant cases are truly definable as Ph+ multilineage/CML-like. This will allow to understand the cellular origin of *BCR::ABL1*.

The objective of the case report description was to highlight that the genomic complexity might, in a few cases, overseed the therapeutic improvement observed in recent years. Therefore, an extensive characterization is required to deepen the knowledge on poor responder's cases. Beyond *IKZF1*^{plus} genotype and the development of an ABL1 *T315I* mutation also in the setting of a ponatinib-based treatment, the highly proliferative features of the patient's disease led to a deeper characterization of his genomic and genetic features and the only additional and "unconventional" lesion identified was represented by the *SDHA* R352* mutation. *SDHA* gene, i.e. succinate dehydrogenase complex flavoprotein subunit A, located on 5p15.33, encodes for a major catalytic subunit of the succinate-ubiquinone oxidoreductase complex II involved in mitochondrial respiratory chain composed by four nuclear-encoded subunit SDHA, SDHB, SDHC, SDHD (SDH-x), included in the oxidative phosphorylation system (OXPHOS). Genetic alterations in complex I and II of the mitochondrial respiratory chain were already known to have a strong association with severe neurologic disorders and syndromes, as well as tumor-suppression function; indeed, mutations in these genes are associated with the development of several types of solid tumors, mostly gastrointestinal stromal tumors (GISTs) and pheochromocytomas. R352* mutation results from a C to T substitution in nucleotide position 1054 in the coding exon 8 of the *SDHA* gene and gives origine to a stop-codon that generates a truncated protein with a consequent loss of function of the enzyme already known to be a disease-causing mutation in GISTs and paragangliomas. Notably, in this case considering the absence of the mutation in germinal DNA, we could conclude that SDHA R352* might be a somatic mutation carried by the leukemic clone. Regarding the hematological malignancies, few works have reported an association with genetic alterations in *SDH-x* genes so far, and at the best of our knowledge, this is the first report of the

involvement of the *SDHA* gene in the Ph+ ALL setting. While screening and expression analysis on additional Ph+ ALL patients are warranted, a similar case was found at the University of Chicago (personal communication from Dr. Caner Saygin) suggesting that this gene might have a pivotal role in progression not only in solid tumors, but also in hematologic neoplasms where it may represent a key driver of leukemia resistance/progression.

In conclusion, the use of ddPCR for the MRD monitoring by using the fusion transcript of *BCR::ABL1* as a marker is at least comparable to the gold standard method represented by RT-qPCR. This is demonstrated by high concordance and correlation rates. Furthermore, the use of ddPCR represents a clear improvement in terms of sensitivity, as it can markedly reduce the number of PNQ cases and recover positivity between cases otherwise considered negative. The IG/TR clonal rearrangements-based MRD monitoring in the Ph+ ALL adult setting is feasible in the 87.4% of cases. However, the overall correlation rate between the two markers is only 43.9%, which is confirmed as suboptimal. Further studies will be needed to understand the real prognostic value of IG/TR. However, in 2/5 patients who experienced a hematologic relapse, the IG/TR marker was a more reliable indicator for revealing an MRD positivity than the *BCR::ABL1*. Despite significant advancements in both clinical and biological fields, there are still patients who exhibit resistance to therapy and who are at risk of relapse and, ultimately, mortality. As described in the clinical case, further investigation at the molecular level is required in order to identify new genetic features that may contribute to the development of the pathology or starting a relapse, and thus becoming potential therapeutic targets. In this case, the identification of the R352* mutation on the *SDHA* gene has, for the first time, revealed the involvement of this gene in Ph+ ALL. However, further functional studies are required to establish the true impact of this mutation and its potential pathogenetic effects.

5 Future perspectives

The primary objective in the next future will be to expand the study population and to increase the number of samples monitored for each patient. This will be possible thanks to the advancement of the GIMEMA ALL2820 clinical trial, which will allow the inclusion of a greater number of patients and the availability of a greater number of samples at later TPs. This will be achieved through the continuation the enrollment and the extension of follow-up for those patients who have been already enrolled. Indeed, the two primary endpoints of the protocol, as studied in this project, will pave the way for extending the MRD monitoring to subsequent stages of treatment. This will allow the availability of more data and enable a more comprehensive analysis to identify which could be the TP with the greatest prognostic value. In light of the existing work on *BCR::ABL1*-based MRD monitoring by ddPCR, as well as the findings of this study, the objective is to contribute to the integration of ddPCR in the clinical practice. This is because the practical and clinical benefits might have reached a sufficient level of maturity, and the methodology, although it requires a definitive standardization, appear relatively "ready to use" in technical and economic terms for the laboratories operating in this field. The NGS and ddPCR approach for IG/TR-based MRD monitoring appears to be promising; however, further studies and increasing the study cohort are required, particularly to obtain significant clinical correlations, in order to establish the real prognostic value of the IG/TR alone or in combination with the *BCR::ABL1* monitoring. From a biological standpoint, the objective is to conduct a more detailed analysis of samples derived from patients exhibiting pronounced discrepancies, particularly in those cases where the levels of the *BCR::ABL1* are markedly higher and persistently positive in comparison to the IG/TR. In these patients, based on the available

biologic material, the aim is to separate different cellular populations to establish whether the *BCR::ABL1* can be present in other cells than the leukemic blasts. From a methodological standpoint, the objective is to investigate the different cellular populations after cell sorting in order to determine, by ddPCR, which cellular compartments express the *BCR::ABL1*. As a further step, based on the available biologic material, a selected cohort will be subjected to single-cell RNA-sequencing in order to identify possible differences in terms of gene expression profile and to define a molecular mark able to identify the Ph+ ALL CML-like/multilineage subgroup. Ultimately, the data will be correlated with clinical features, treatment response, outcome (OS, DFS, relapse incidence), and additional genomic/genetic characteristics to assess the potential direct impact of this phenomenon on the management of Ph+ ALL adult patients.

References

1. Jabbour, E., O'Brien, S., Konopleva, M. & Kantarjian, H. New insights into the pathophysiology and therapy of adult acute lymphoblastic leukemia. *Cancer* **121**, 2517–2528 (2015).

2. Jemal, A. *et al.* Cancer statistics, 2004. *CA. Cancer J. Clin.* **54**, 8–29 (2004).

3. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2015. *CA. Cancer J. Clin.* **65**, 5–29 (2015).

4. Chiaretti, S. *et al.* Clinico-biological features of 5202 patients with acute lymphoblastic leukemia enrolled in the Italian AIEOP and GIMEMA protocols and stratified in age cohorts. *Haematologica* **98**, 1702–1710 (2013).

5. Paul, S., Kantarjian, H. & Jabbour, E. J. Adult Acute Lymphoblastic Leukemia. *Mayo Clin. Proc.* **91**, 1645–1666 (2016).

6. Perez-Andreu, V. *et al.* Inherited GATA3 variants are associated with Ph-like childhood acute lymphoblastic leukemia and risk of relapse. *Nat. Genet.* **45**, 1494–1498 (2013).

7. Treviño, L. R. *et al.* Germline genomic variants associated with childhood acute lymphoblastic leukemia. *Nat. Genet.* **41**, 1001–1005 (2009).

8. Papaemmanuil, E. *et al.* Loci on 7p12.2, 10q21.2 and 14q11.2 are associated with risk of childhood acute lymphoblastic leukemia. *Nat. Genet.* **41**, 1006–1010 (2009).

9. Shah, S. *et al.* A recurrent germline PAX5 mutation confers susceptibility to pre-B cell acute lymphoblastic leukemia. *Nat. Genet.* **45**, 1226–1231 (2013).

10. Zhang, M. Y. *et al.* Germline ETV6 mutations in familial thrombocytopenia and hematologic malignancy. *Nat. Genet.* **47**, 180–185 (2015).

11. Hunger, S. P. & Mullighan, C. G. Acute Lymphoblastic Leukemia in Children. *N. Engl. J. Med.* **373**, 1541–1552 (2015).

12. Inaba, H., Greaves, M. & Mullighan, C. G. Acute lymphoblastic leukaemia. *Lancet (London, England)* **381**, 1943–1955 (2013).

13. Haferlach, T. *et al.* Global approach to the diagnosis of leukemia using gene expression profiling. *Blood* **106**, 1189–1198 (2005).

14. Chiaretti, S. *et al.* Gene expression profiles of B-lineage adult acute lymphocytic leukemia reveal genetic patterns that identify lineage derivation and distinct mechanisms of transformation. *Clin. cancer Res. an Off. J. Am. Assoc. Cancer Res.* **11**, 7209–7219 (2005).

15. Haferlach, T. *et al.* Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the International Microarray Innovations in Leukemia Study Group. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **28**, 2529–2537 (2010).

16. Mullighan, C. G. *et al.* Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* **446**, 758–764 (2007).

17. Iacobucci, I., Kimura, S. & Mullighan, C. G. Biologic and therapeutic implications of genomic alterations in acute lymphoblastic leukemia. *J. Clin. Med.* **10**, (2021).

18. Alaggio, R. *et al.* The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms. *Leukemia* **36**, 1720–1748 (2022).

19. Chiaretti, S., Zini, G. & Bassan, R. Diagnosis and subclassification of acute lymphoblastic leukemia. *Mediterr. J. Hematol. Infect. Dis.* **6**, e2014073 (2014).

20. Arber, D. A. *et al.* The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* **127**, 2391–

2405 (2016).

21. Hoelzer, D. *et al.* Improved outcome of adult Burkitt lymphoma/leukemia with rituximab and chemotherapy: report of a large prospective multicenter trial. *Blood* **124**, 3870–3879 (2014).

22. Szczepański, T., van der Velden, V. H. J. & van Dongen, J. J. M. Flow-cytometric immunophenotyping of normal and malignant lymphocytes. *Clin. Chem. Lab. Med.* **44**, 775–796 (2006).

23. Kalina, T. *et al.* EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia* **26**, 1986–2010 (2012).

24. Béné, M. C. *et al.* Immunophenotyping of acute leukemia and lymphoproliferative disorders: a consensus proposal of the European LeukemiaNet Work Package 10. *Leukemia* **25**, 567–574 (2011).

25. Vitale, A. *et al.* Absence of prognostic impact of CD13 and/or CD33 antigen expression in adult acute lymphoblastic leukemia. Results of the GIMEMA ALL 0496 trial. *Haematologica* **92**, 342–348 (2007).

26. Bueno, C., Montes, R., Catalina, P., Rodríguez, R. & Menendez, P. Insights into the cellular origin and etiology of the infant pro-B acute lymphoblastic leukemia with MLL-AF4 rearrangement. *Leukemia* **25**, 400–410 (2011).

27. Coustan-Smith, E. *et al.* Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. *Lancet. Oncol.* **10**, 147–156 (2009).

28. Weinberg, O. K. *et al.* Adult mixed phenotype acute leukemia (MPAL): B/myeloid MPAL(isoMPO) is distinct from other MPAL subtypes. *Int. J. Lab. Hematol.* **45**, 170–178 (2023).

29. Laganà, A. *et al.* CD146 Molecule Expression in B Cells Acute Lymphoblastic Leukemia (B-ALLs): A Flow-Cytometric Marker for an

Accurate Diagnostic Workup. *Mediterr. J. Hematol. Infect. Dis.* **16**, e2024064 (2024).

30. Arber, D. A. *et al.* International Consensus Classification of Myeloid Neoplasms and Acute Leukemias: integrating morphologic, clinical, and genomic data. *Blood* **140**, 1200–1228 (2022).

31. Kamoda, Y. *et al.* Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia Is Separated into Two Subgroups Associated with Survival by BCR-ABL Fluorescence in situ Hybridization of Segmented Cell Nuclei: Report from a Single Institution. *Acta Haematol.* **136**, 157–166 (2016).

32. Chen, Z. *et al.* Chronic myeloid leukemia presenting in lymphoblastic crisis, a differential diagnosis with Philadelphia-positive B-lymphoblastic leukemia. *Leuk. Lymphoma* **61**, 2831–2838 (2020).

33. Hovorkova, L. *et al.* Monitoring of childhood ALL using BCR-ABL1 genomic breakpoints identifies a subgroup with CML-like biology. *Blood* **129**, 2771–2781 (2017).

34. Meyer, C. *et al.* The MLL recombinome of acute leukemias in 2013. *Leukemia* **27**, 2165–2176 (2013).

35. Harrison, C. J. Cytogenetics of paediatric and adolescent acute lymphoblastic leukaemia. *Br. J. Haematol.* **144**, 147–156 (2009).

36. Hunger, S. P. Chromosomal translocations involving the E2A gene in acute lymphoblastic leukemia: clinical features and molecular pathogenesis. *Blood* **87**, 1211–1224 (1996).

37. Chiaretti, S. *et al.* Rapid identification of BCR/ABL1-like acute lymphoblastic leukaemia patients using a predictive statistical model based on quantitative real time-polymerase chain reaction: clinical, prognostic and therapeutic implications. *Br. J. Haematol.* **181**, 642–652

(2018).

38. Kaiser, F. *et al.* Ponatinib alone or with chemo-immunotherapy in heavily pre-treated Philadelphia-like acute lymphoblastic leukemia: a CAMPUS ALL real-life study. *Haematologica* (2024) doi:10.3324/haematol.2024.285258.

39. Holmfeldt, L. *et al.* The genomic landscape of hypodiploid acute lymphoblastic leukemia. *Nat. Genet.* **45**, 242–252 (2013).

40. Yasuda, T. *et al.* Recurrent DUX4 fusions in B cell acute lymphoblastic leukemia of adolescents and young adults. *Nat. Genet.* **48**, 569–574 (2016).

41. Shinsuke Hirabayashi *et al.* ZNF384-related fusion genes define a subgroup of childhood B-cell precursor acute lymphoblastic leukemia with a characteristic immunotype. *Haematologica* **102**, 118–129 (2016).

42. Hirabayashi, S. *et al.* Clinical characteristics and outcomes of B-ALL with ZNF384 rearrangements: a retrospective analysis by the Ponte di Legno Childhood ALL Working Group. *Leukemia* **35**, 3272–3277 (2021).

43. Chiaretti, S. *et al.* ZNF384 rearrangement is the most frequent genetic lesion in adult PH-negative and Ph-like-negative B-other acute lymphoblastic leukemia. Biological and clinical findings. *Leuk. Lymphoma* **64**, 483–486 (2023).

44. Gu, Z. *et al.* Genomic analyses identify recurrent MEF2D fusions in acute lymphoblastic leukaemia. *Nat. Commun.* **7**, 13331 (2016).

45. Wagener, R. *et al.* IG-MYC (+) neoplasms with precursor B-cell phenotype are molecularly distinct from Burkitt lymphomas. *Blood* **132**, 2280–2285 (2018).

46. Gu, Z. *et al.* PAX5-driven subtypes of B-progenitor acute lymphoblastic leukemia. *Nat. Genet.* **51**, 296–307 (2019).

47. Kantarjian, H. *et al.* Long-term follow-up results of hyperfractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone (Hyper-CVAD), a dose-intensive regimen, in adult acute lymphocytic leukemia. *Cancer* **101**, 2788–2801 (2004).

48. Thomas, X. *et al.* Outcome of treatment in adults with acute lymphoblastic leukemia: analysis of the LALA-94 trial. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **22**, 4075–4086 (2004).

49. Rowe, J. M. *et al.* Induction therapy for adults with acute lymphoblastic leukemia: results of more than 1500 patients from the international ALL trial: MRC UKALL XII/ECOG E2993. *Blood* **106**, 3760–3767 (2005).

50. Foà, R. & Chiaretti, S. Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia. *N. Engl. J. Med.* **386**, 2399–2411 (2022).

51. Score, J. *et al.* Analysis of genomic breakpoints in p190 and p210 BCR-ABL indicate distinct mechanisms of formation. *Leukemia* **24**, 1742–1750 (2010).

52. Quintás-Cardama, A., Kantarjian, H. & Cortes, J. Flying under the radar: The new wave of BCR-ABL inhibitors. *Nat. Rev. Drug Discov.* **6**, 834–848 (2007).

53. Li, S., Ilaria, R. L. J., Million, R. P., Daley, G. Q. & Van Etten, R. A. The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. *J. Exp. Med.* **189**, 1399–1412 (1999).

54. Kang, Z. J. *et al.* The philadelphia chromosome in leukemogenesis. *Chin. J. Cancer* **35**, 1–15 (2016).

55. Cramer, K. *et al.* BCR/ABL and other kinases from chronic myeloproliferative disorders stimulate single-strand annealing, an unfaithful DNA double-strand break repair. *Cancer Res.* **68**, 6884–6888

(2008).

56. Theocharides, A. P. A. *et al.* Dominant-negative Ikaros cooperates with BCR-ABL1 to induce human acute myeloid leukemia in xenografts. *Leukemia* **29**, 177–187 (2015).

57. Mullighan, C. G. *et al.* BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature* **453**, 110–114 (2008).

58. Fedullo, A. L. *et al.* Prognostic implications of additional genomic lesions in adult Philadelphia chromosome-positive acute lymphoblastic leukemia. *Haematologica* **104**, 312–318 (2019).

59. Jaso, J. *et al.* Prognostic significance of immunophenotypic and karyotypic features of Philadelphia positive B-lymphoblastic leukemia in the era of tyrosine kinase inhibitors. *Cancer* **117**, 4009–4017 (2011).

60. Rowe, J. M. Optimal management of adults with ALL. *Br. J. Haematol.* **144**, 468–483 (2009).

61. Fielding, A. K. The treatment of adults with acute lymphoblastic leukemia. *Hematol. Am. Soc. Hematol. Educ. Progr.* 381–389 (2008) doi:10.1182/asheducation-2008.1.381.

62. Chiaretti, S. *et al.* A sequential approach with imatinib, chemotherapy and transplant for adult Ph+ acute lymphoblastic leukemia: final results of the GIMEMA LAL 0904 study. *Haematologica* **101**, 1544–1552 (2016).

63. Chalandon, Y. *et al.* Randomized study of reduced-intensity chemotherapy combined with imatinib in adults with Ph-positive acute lymphoblastic leukemia. *Blood* **125**, 3711–3719 (2015).

64. Bassan, R. *et al.* Chemotherapy-phased imatinib pulses improve long-term outcome of adult patients with Philadelphia chromosome-positive acute lymphoblastic leukemia: Northern Italy Leukemia Group protocol 09/00. *J.*

Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. **28**, 3644–3652 (2010).

65. Druker, B. J. *et al.* Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N. Engl. J. Med.* **344**, 1031–1037 (2001).

66. Druker, B. J. *et al.* Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat. Med.* **2**, 561–566 (1996).

67. Gorre, M. E. *et al.* Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* **293**, 876–880 (2001).

68. Gambacorti-Passerini, C. B. *et al.* Molecular mechanisms of resistance to imatinib in Philadelphia-chromosome-positive leukaemias. *Lancet. Oncol.* **4**, 75–85 (2003).

69. Nagar, B. *et al.* Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). *Cancer Res.* **62**, 4236–4243 (2002).

70. Skaggs, B. J. *et al.* Phosphorylation of the ATP-binding loop directs oncogenicity of drug-resistant BCR-ABL mutants. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 19466–19471 (2006).

71. Azam, M., Latek, R. R. & Daley, G. Q. Mechanisms of autoinhibition and STI-571/imatinib resistance revealed by mutagenesis of BCR-ABL. *Cell* **112**, 831–843 (2003).

72. Ribera, J.-M. *et al.* Concurrent intensive chemotherapy and imatinib before and after stem cell transplantation in newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia. Final results of the CSTIBES02 trial. *Haematologica* **95**, 87–95 (2010).

73. Vignetti, M. *et al.* Imatinib plus steroids induces complete remissions and prolonged survival in elderly Philadelphia chromosome-positive patients

with acute lymphoblastic leukemia without additional chemotherapy: results of the Gruppo Italiano Malattie Ematologiche dell'Ad. *Blood* **109**, 3676–3678 (2007).

74. Short, N. J. *et al.* Impact of complete molecular response on survival in patients with Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood* **128**, 504–507 (2016).

75. Foà, R. *et al.* Dasatinib–Blinatumomab for Ph-Positive Acute Lymphoblastic Leukemia in Adults. *N. Engl. J. Med.* **383**, 1613–1623 (2020).

76. Foà, R. *et al.* Long-Term Results of the Dasatinib-Blinatumomab Protocol for Adult Philadelphia-Positive ALL. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **42**, 881–885 (2024).

77. Martinelli, G. *et al.* Complete Hematologic and Molecular Response in Adult Patients With Relapsed/Refractory Philadelphia Chromosome-Positive B-Precursor Acute Lymphoblastic Leukemia Following Treatment With Blinatumomab: Results From a Phase II, Single-Arm, Multicenter Study. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **35**, 1795–1802 (2017).

78. Park, J. H. *et al.* Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. *N. Engl. J. Med.* **378**, 449–459 (2018).

79. Pui, C.-H. Central nervous system disease in acute lymphoblastic leukemia: prophylaxis and treatment. *Hematol. Am. Soc. Hematol. Educ. Progr.* 142–146 (2006) doi:10.1182/asheducation-2006.1.142.

80. Chiaretti, S. *et al.* P353: FORTY MONTHS UPDATE OF THE GIMEMA LAL2116 (D-ALBA) PROTOCOL AND ANCILLARY LAL2217 STUDY FOR NEWLY DIAGNOSED ADULT PH+ ALL. *HemaSphere* **6**, (2022).

81. Della Starza, I. *et al.* Minimal Residual Disease in Acute Lymphoblastic Leukemia: Technical and Clinical Advances. *Front. Oncol.* **9**, 726 (2019).

82. Raff, T. *et al.* Molecular relapse in adult standard-risk ALL patients detected by prospective MRD monitoring during and after maintenance treatment: data from the GMALL 06/99 and 07/03 trials. *Blood* **109**, 910–915 (2007).

83. Borowitz, M. J. *et al.* Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: a Children's Oncology Group study. *Blood* **111**, 5477–5485 (2008).

84. Gökbuget, N. *et al.* Adult patients with acute lymphoblastic leukemia and molecular failure display a poor prognosis and are candidates for stem cell transplantation and targeted therapies. *Blood* **120**, 1868–1876 (2012).

85. Elia, L. *et al.* A multiplex reverse transcriptase-polymerase chain reaction strategy for the diagnostic molecular screening of chimeric genes: a clinical evaluation on 170 patients with acute lymphoblastic leukemia. *Haematologica* **88**, 275–279 (2003).

86. van Dongen, J. J. M. *et al.* Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. *Leukemia* **13**, 1901–1928 (1999).

87. Gabert, J. *et al.* Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. *Leukemia* **17**, 2318–2357 (2003).

88. Pfeifer, H. *et al.* Standardisation and consensus guidelines for minimal residual disease assessment in Philadelphia-positive acute lymphoblastic leukemia (Ph + ALL) by real-time quantitative reverse transcriptase PCR of e1a2 BCR-ABL1. *Leukemia* **33**, 1910–1922 (2019).

89. Hovorkova, L. *et al.* Distinct pattern of genomic breakpoints in CML and BCR::ABL1-positive ALL: analysis of 971 patients. *Mol. Cancer* **23**, 138 (2024).

90. van der Velden, V. H. J. *et al.* Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. *Leukemia* **21**, 604–611 (2007).

91. van der Velden, V. H. J. *et al.* Analysis of measurable residual disease by IG/TR gene rearrangements: quality assurance and updated EuroMRD guidelines. *Leukemia* **38**, 1315–1322 (2024).

92. Kotrova, M., Darzentas, N., Pott, C., Baldus, C. D. & Brüggemann, M. Immune Gene Rearrangements: Unique Signatures for Tracing Physiological Lymphocytes and Leukemic Cells. *Genes (Basel).* **12**, (2021).

93. Tonegawa, S. Somatic generation of antibody diversity. *Nature* **302**, 575–581 (1983).

94. Jung, D., Giallourakis, C., Mostoslavsky, R. & Alt, F. W. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Annu. Rev. Immunol.* **24**, 541–570 (2006).

95. Saygin, C., Cannova, J., Stock, W. & Muffly, L. Measurable residual disease in acute lymphoblastic leukemia: methods and clinical context in adult patients. *Haematologica* **107**, 2783–2793 (2022).

96. Della Starza, I. *et al.* Optimizing Molecular Minimal Residual Disease Analysis in Adult Acute Lymphoblastic Leukemia. *Cancers (Basel).* **15**, 1–16 (2023).

97. Kotrova, M. *et al.* The gray area of RQ-PCR-based measurable residual disease: subdividing the "positive, below quantitative range" category. *Leukemia* **38**, 1617–1620 (2024).

98. Huggett, J. F. & Whale, A. Digital PCR as a novel technology and its potential implications for molecular diagnostics. *Clinical chemistry* vol. 59 1691–1693 (2013).

99. Whale, A. S., Cowen, S., Foy, C. A. & Huggett, J. F. Methods for applying accurate digital PCR analysis on low copy DNA samples. *PLoS One* **8**, e58177 (2013).

100. Dube, S., Qin, J. & Ramakrishnan, R. Mathematical analysis of copy number variation in a DNA sample using digital PCR on a nanofluidic device. *PLoS One* **3**, e2876 (2008).

101. Albano, F. *et al.* Absolute quantification of the pretreatment PML-RARA transcript defines the relapse risk in acute promyelocytic leukemia. *Oncotarget* **6**, 13269–13277 (2015).

102. Jennings, L. J., George, D., Czech, J., Yu, M. & Joseph, L. Detection and quantification of BCR-ABL1 fusion transcripts by droplet digital PCR. *J. Mol. Diagn.* **16**, 174–179 (2014).

103. Goh, H.-G. *et al.* Sensitive quantitation of minimal residual disease in chronic myeloid leukemia using nanofluidic digital polymerase chain reaction assay. *Leuk. Lymphoma* **52**, 896–904 (2011).

104. Drandi, D. *et al.* Minimal Residual Disease Detection by Droplet Digital PCR in Multiple Myeloma, Mantle Cell Lymphoma, and Follicular Lymphoma: A Comparison with Real-Time PCR. *J. Mol. Diagn.* **17**, 652–660 (2015).

105. Stahl, T. *et al.* Digital PCR Panel for Sensitive Hematopoietic Chimerism Quantification after Allogeneic Stem Cell Transplantation. *Int. J. Mol. Sci.* **17**, (2016).

106. Cardinali, D. *et al.* Digital droplet PCR for T315I BCR::ABL1 KD mutation

assessment in adult Ph-positive acute lymphoblastic leukemia with a minimal residual disease increase. *Leuk. Lymphoma* **64**, 1884–1887 (2023).

107. van Dongen, J. J. M., van der Velden, V. H. J., Brüggemann, M. & Orfao, A. Minimal residual disease diagnostics in acute lymphoblastic leukemia: need for sensitive, fast, and standardized technologies. *Blood* **125**, 3996–4009 (2015).

108. Contreras Yametti, G. P. *et al.* Minimal Residual Disease in Acute Lymphoblastic Leukemia: Current Practice and Future Directions. *Cancers (Basel).* **13**, (2021).

109. Wu, D. *et al.* High-throughput sequencing detects minimal residual disease in acute T lymphoblastic leukemia. *Sci. Transl. Med.* **4**, 134ra63 (2012).

110. Kotrova, M. *et al.* The predictive strength of next-generation sequencing MRD detection for relapse compared with current methods in childhood ALL. *Blood* **126**, 1045–1047 (2015).

111. Zuna, J. *et al.* Minimal residual disease in BCR::ABL1-positive acute lymphoblastic leukemia: different significance in typical ALL and in CML-like disease. *Leukemia* **36**, 2793–2801 (2022).

112. Cazzaniga, G. *et al.* Predictive value of minimal residual disease in Philadelphia-chromosome-positive acute lymphoblastic leukemia treated with imatinib in the European intergroup study of post-induction treatment of Philadelphia-chromosome-positive acute lymphoblastic leuke. *Haematologica* **103**, 107–115 (2018).

113. Short, N. J. *et al.* Ultrasensitive NGS MRD assessment in Ph+ ALL: Prognostic impact and correlation with RT-PCR for BCR::ABL1. *Am. J. Hematol.* **98**, 1196–1203 (2023).

114. Kim, R. *et al.* Significance of Measurable Residual Disease in Adult

Philadelphia Chromosome-Positive ALL: A GRAAPH-2014 Study. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **42**, 3140–3150 (2024).

115. Kim, J. C. *et al.* Transcriptomic classes of BCR-ABL1 lymphoblastic leukemia. *Nat. Genet.* **55**, 1186–1197 (2023).

116. Bastian, L. *et al.* Developmental trajectories and cooperating genomic events define molecular subtypes of BCR::ABL1-positive ALL. *Blood* **143**, 1391–1398 (2024).

117. Chiaretti, S. *et al.* Comparison between Dasatinib-Blinatumomab Vs Ponatinib-Blinatumomab Chemo-Free Strategy for Newly Diagnosed Ph+ Acute Lymphoblastic Leukemia Patients. Preliminary Results of the Gimema ALLL2820 Trial. *Blood* **142**, 4249 (2023).

118. van Dongen, J. J. M. *et al.* Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* **17**, 2257–2317 (2003).

119. Pongers-Willemse, M. J. *et al.* Primers and protocols for standardized detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin and T cell receptor gene rearrangements and TAL1 deletions as PCR targets: report of the BIOMED-1 CONCERTED ACTION: investig. *Leukemia* **13**, 110–118 (1999).

120. Paulsen, K., Marincevic, M., Cavelier, L., Hollander, P. & Amini, R.-M. LymphoTrack Is Equally Sensitive as PCR GeneScan and Sanger Sequencing for Detection of Clonal Rearrangements in ALL Patients. *Diagnostics (Basel, Switzerland)* **12**, (2022).

121. Ho, C. C., Tung, J. K., Zehnder, J. L. & Zhang, B. M. Validation of a Next-Generation Sequencing-Based T-Cell Receptor Gamma

Gene Rearrangement Diagnostic Assay: Transitioning from Capillary Electrophoresis to Next-Generation Sequencing. *J. Mol. Diagn.* **23**, 805–815 (2021).

122. Ansuinelli, M. *et al.* Applicability of droplet digital polymerase chain reaction for minimal residual disease monitoring in Philadelphia-positive acute lymphoblastic leukaemia. *Hematol. Oncol.* **39**, 680–686 (2021).

123. Schwinghammer, C. *et al.* A New View on Minimal Residual Disease Quantification in Acute Lymphoblastic Leukemia using Droplet Digital PCR. *J. Mol. Diagnostics* **24**, 856–866 (2022).

124. Benard-Slagter, A. *et al.* Digital Multiplex Ligation-Dependent Probe Amplification for Detection of Key Copy Number Alterations in T- and B-Cell Lymphoblastic Leukemia. *J. Mol. Diagnostics* **19**, 659–672 (2017).

125. Thakral, D. *et al.* Rapid Identification of Key Copy Number Alterations in B- and T-Cell Acute Lymphoblastic Leukemia by Digital Multiplex Ligation-Dependent Probe Amplification. *Front. Oncol.* **9**, 871 (2019).

126. Lilljebjörn, H. *et al.* Whole-exome sequencing of pediatric acute lymphoblastic leukemia. *Leukemia* **26**, 1602–1607 (2012).