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NELLINA MARIA AUSILIA ANDRIANO

Role of the DNA Repair Machinery in Childhood B-Lineage Acute Lymphoblastic Leukemia: Aberrations of NBS1, Fancd2, Palb2 Genes and Expression of BRCA1 and BRCA2

Doctoral thesis

Supervisor:

PROF. CARLO VANCHERI

Coordinator:

PROF.ssa GIOVANNA RUSSO

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Abstract

Successful treatment of B-lineage Acute Lymphoblastic Leukemia (B-ALL) in children has been related to novel biological findings and a better supportive therapy. Genomic aberrations have a major role in development of ALL and in treatment response. Conventional treatment is based on chemotherapy, mainly acting on the structure of DNA in both leukemic and host cells. The result is a complete achievement of durable remission and/or a high incidence of toxicity. The role of DNA repair machinery in the occurrence of de novo or relapsed ALL and of high-grade toxicity is still unclear. We decide to dissect genes involved in the Fanconi Anemia (FA) pathway (BRCA1, BRCA2, FANCD2 and PALB2), which cooperates with the Nijmegen breakage syndrome (NBS1) gene. We evaluate the correlation of DNA repair machinery alterations with occurrence of relapse and/or with severe (III-IV grade – CTCAE) toxicity during chemotherapy treatment.

The study was approved by the Local Clinical Research Ethics Committee (Catania-1). Written informed consents were obtained from the parents of each child involved in this research. We analyzed bone marrow (BM) samples from diagnosis and remission (Dx/Rem) of 111 children with B-ALL, diagnosed and treated at the Center of Pediatric Hematology Oncology, Catania Italy, from 2000 to 2017. These children were enrolled in three consecutive protocols of Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP) [AIEOP-BFM ALL 2000; AIEOP-R 2006; AIEOP BFM ALL 2009]. Biological subgroups were as follow: 16 children presenting with t(1;19); 48 with t(12;21), 9 with t(9;22)/Ph+, 5 with a KMT2A (formerly MLL) rearrangement and 33 defined as “B-Others”. Traditional PCR method was used to determinate the NBS1 mutations in exons 3-6, followed by Sanger sequencing. FANCD2 and PALB2 mutations were investigated by multiplex ligation-dependent probe amplification (MLPA). Samples from healthy donors were used as wild-type controls. A real-time PCR assay was applied to evaluate the BRCA1 and BRCA2 genes expression using the SYBR green, fluorescent dye. We used specific primers for BRCA1 exons 14-15 and BRCA2 exons 15-16, respectively. The expression of BRCA1 and BRCA2 was determined as high (H) or low (L) using the Mean and the Median of fold change (FC) as cut-off.

Fifty-four patients (48%) out of 111 had more than one episode of severe toxicity during chemotherapy treatment. Twenty-one children (19%) with B-ALL out of 111 experienced the disease recurrence. The NBS1 polymorphism (rs1805794 SNP_G>C), was found in 28% of the patients enrolled in the study and showed a protective role against severe toxicity ($p<0.05$): only 10 of 54 patients, who experienced high grade toxicity, had this polymorphism. This protective effect was confirmed in the subgroup with t(12;21) positive B-ALL ($p<0.05$). There were no statistically significant relationships between NBS1 and relapse or between FANCD2/PALB2 aberrations and B-ALL toxicity/recurrence. At diagnosis, children with high expression of both BRCA genes (BRCA1-H/BRCA2-H) showed a correlation with an increased incidence of severe toxicity: 17 patients with severe toxicity out of 27 BRCA1-H/BRCA2-H cases vs 10 out of 28 BRCA1L/BRCA2-L ($p<0.05$). At remission, high BRCA1 expression (BRCA1-H) appears to be related to an increased risk of relapse, albeit not statistically significant ($p=0.17$). BRCA2-H is associated with a higher incidence of recurrence than BRCA2-L ($p<0.03$): 8 cases with relapse out of 22 (BRCA2H) vs 13 out of 88 (BRCA2-L). More importantly, we observed that cases with BRCA1-H/BRCA2-H are statistically significant associated with relapse: 3 children presented a relapse out of 6 cases with BRCA1-H/BRCA2-H vs 8 out of 65 cases with BRCA1-L/BRCA2-L($p<0.02$).

Our study strongly demonstrates that the NBS1 gene polymorphism (rs1805794 SNP_G>C) has a protective role against toxicity, reducing the incidence of severe grade episodes. High expression of both BRCA1 and BRCA2 genes in leukemic cells seems to be associated with a higher incidence of severe toxicity. Finally, high expression of BRCA1 and BRCA2 genes in the host cells, at remission, is strongly correlated with a higher risk of relapse.

Although this study has been performed with an adequate number of children with B-ALL, it is mandatory to perform a prospective project with a larger population

Table of contents

Chapter 1. Acute Lymphoblastic Leukemia

INTRODUCTION

Acute Lymphoblastic Leukemia (ALL) is the most common cancer in children (*Airtum 2013*). Observational epidemiology has demonstrated that about 5 to 10% of ALL are associated with ionizing radiation exposure and congenital genetic syndromes (Down, neurofibromatosis, Fanconi anemia, and Bloom syndrome) (*Greaves 2018*). Nowadays ALL is deeply characterized at the cytogenetic and molecular level (*Lo Nigro 2013, Iacobucci 2017*). However the mechanism of leukemogenesis is still unclear and it is likely to have different characteristics in association with different molecular subtypes (*Lo Nigro 2013, Iacobucci 2017*). Moreover, it has been clearly demonstrated that specific chromosomal translocations (*ETV*6-*RUNX*1; *BCR-ABL*1) arise *in utero* or remain detectable after suspension of therapy, strongly suggesting a sort of predisposition to genetic aberrations (*Greaves 2018, Cazzaniga 2011, Hovorkova 2017*). Indeed, several studies of familial leukemia highlighted the genetic predisposition to childhood ALL (*Stieglitz-Loh 2013*).

In particular, DNA gene repair syndromes are associated with a high incidence of acute leukemias and other types of cancer (*Stieg-Loh 2013*). Germline mutations in cancer-predisposing genes were identified in 8.5% of children and adolescents with cancer (*Zhang 2015*). Family history did not predict the presence of an underlying predisposition syndrome in most patients (*Zhang 2015*). Genes involved in the Fanconi Anemia (FA) pathway (*BRCA*1, *BRCA*2, *FANCD*2 and *PALB*2) cooperate with the Nijmegen breakage syndrome (*NBS*1) gene, repairing the DNA damage with different mechanisms (*Stiegl-Loh 2013; KIM-D'Andrea 2012*). Aberrations of these genes could be related to the occurrence of acute leukemia or involved in the mechanism of response to conventional chemotherapy (*Nalepa-Clapp 2018*). In fact, there are evidences that oncogenic fusion transcript, as *BCR-ABL*1, interferes with the FA/BRCA pathway, altering the formation of FANCD2 foci (*Valeri 2010*).

Other studies suggest that cancer cells become resistant to conventional therapy by relying on certain DNA repair pathways (*Kim-D'Andrea 2012*). Thus, inhibiting the FA pathway may provide a strategy for re-sensitizing resistant cancer cells (*Kee 2012*). Moreover, germline aberrations of the FA pathway and *NBS*1 gene could be associated with a higher incidence of toxic events among children treated with conventional chemotherapy protocols, as curing ALL in familial syndromes demonstrated (*Stieglitz-Loh 2013*).

However, it is less known the role of DNA repair machinery in the activity of pediatric ALL.

Moreover, it is unclear the role of several polymorphisms in genes regulating the FA pathway. Among different components, *BRCA*1 promotes homologous recombination, whereas *BRCA*2 cooperates with *FANCD*2 in the formation of large nuclear foci in response to DNA damage or replication arrest (*D'Andrea 2003*). *NBS*1 has a crucial role in DNA damage response as demonstrated by several studies on familial leukemia (*Stieglitz-Loh 2013*) and its aberrations are associated with childhood ALL (*Mosor 2006; Jiang L 2011*).

Based on the fact that cytogenetic aberrations in ALL could be considered as a result of a genetic instability and FA pathway in association with *NBS*1 regulates the DNA integrity (*Kim-D'Andrea 2012*), we decided to characterize the expression of *BRCA*1 and *BRCA*2, the mutational status of *FANCD*2, *PALB*2 and *NBS*1 in selected cytogenetic and molecular subtypes of pediatric ALL, in order to determine the role of this crucial pathway in different biological subgroups of children with ALL.

1.1. Genetic predisposition to pediatric leukemias

Genetic predisposition to pediatric leukemias is a topic of significant interest in medical research. Germline mutations in certain genes have been associated with an increased risk of developing leukemia in childhood, as mentioned in the study by *Stiegliez et al.* These mutations can elevate the risk even in the absence of other significant clinical manifestations. Understanding the molecular basis of these mutations is crucial for gaining a better understanding of leukemia in children and adolescents, given that leukemia represents the most frequent cancer among individuals under the age of 20, as depicted in Figure 1. However, the frequency of these germline mutations and their implications remains largely unknown [55].

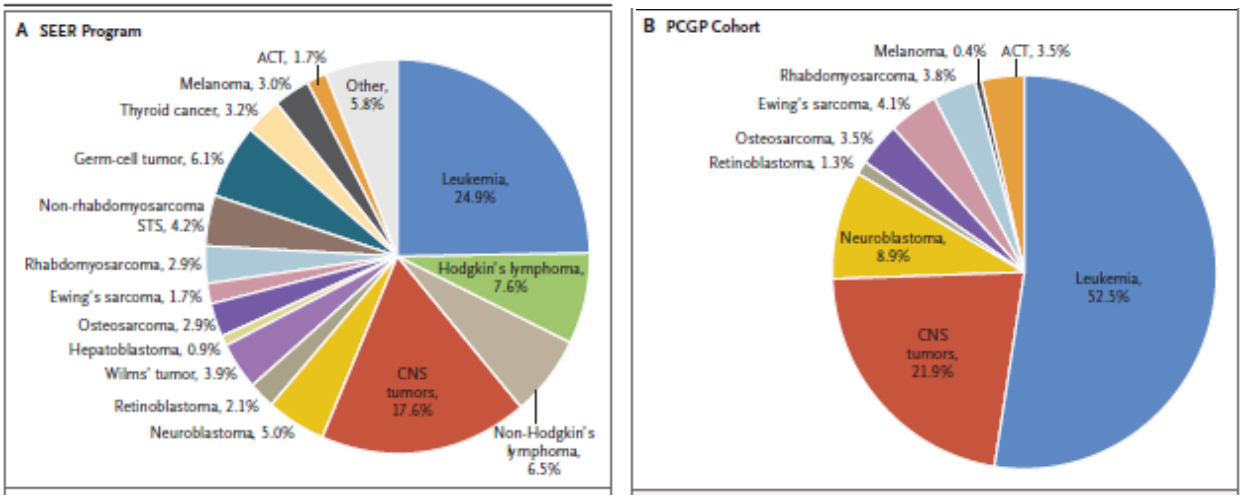


Figure 1. Frequency of Pediatric Cancer Types among Patients Younger than 20 Years of Age Panel A shows the distribution of pediatric cancer types on the basis of data from the Surveillance, Epidemiology, and End Results (SEER) program. Panel B shows the distribution of cancer types analyzed by the Pediatric Cancer Genome Project (PCGP). ACT denotes adrenocortical tumor, CNS central nervous system, and STS soft-tissue sarcoma.

J. Zhang et al., Germline Mutations in Predisposition Genes in Pediatric Cancer. N Engl J Med 2015

Moreover, a total of 565 genes were analyzed and categorized into five groups (Figure 2) [55]. The first category includes genes associated with autosomal dominant syndromes, with 11 of them linked to RAS mutations (syndromes with these types of mutations are also referred to as RASopathies). These 60 genes were selected for their potential impact of germline mutations on clinical decisions, enabling the avoidance of radiation therapy, the choice of a surgical approach for tumor resection, and the testing and selection of donors for stem cell transplantation, with potential or proven benefits for tumor survival or early tumor identification.

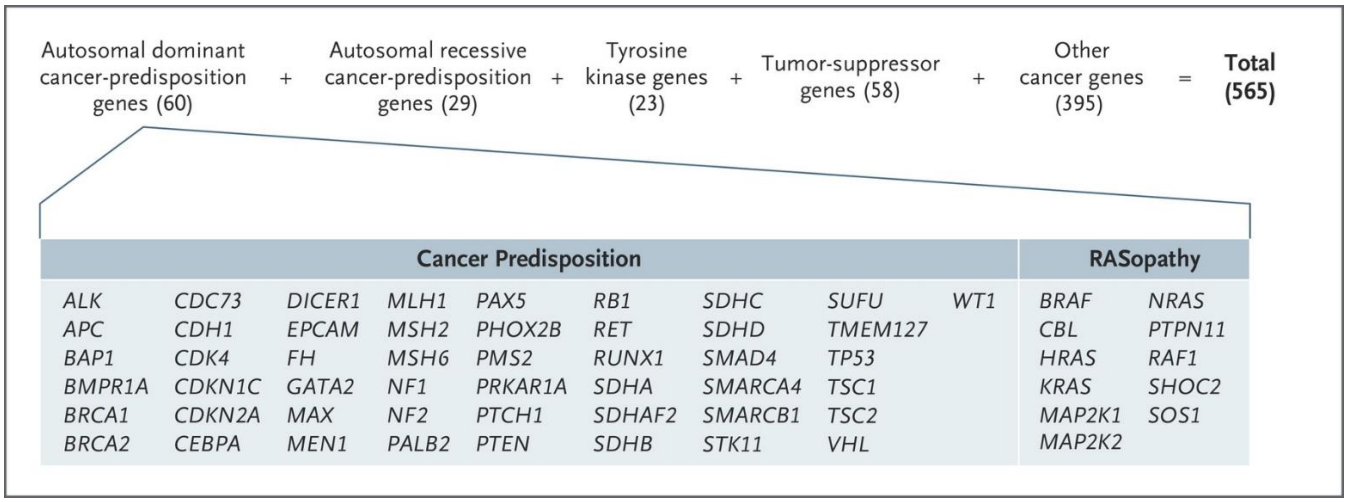


Figure 2. Categories of the 565 Cancer Genes Analyzed for Germline Mutations.

The number of genes in each category is shown in parentheses. Genes that have overlapping categories are listed only once. RASopathies are genetic syndromes that include the cardiofaciocutaneous syndrome, Costello's syndrome (also called the faciocutaneoskeletal syndrome), Noonan's syndrome, and the multiple lentigines syndrome.

J. Zhang et al., Germline Mutations in Predisposition Genes in Pediatric Cancer. N Engl J Med 2015

1.2. Childhood leukemia

Leukemia is a neoplasm characterized by abnormal and uncontrolled expansion of immature cells originating from lymphoid or myeloid precursors [1] or, as recently proposed, from a population of transformed hematopoietic stem cells called leukemia initiating cells [2] . These cells undergo clonal proliferation and infiltrate the peripheral blood and bone marrow, displacing healthy cells. Indeed, the leukemic population replaces the normal marrow components of the erythroid, myeloid, and platelet series and interferes with the remaining normal stem cell component, resulting in severe bone marrow dysfunction, concomitant release of neoplastic elements into the peripheral blood, and leukemic infiltration of parenchyma and tissues [3]. Marrow failure and the presence of immature hematopoietic elements, known as blasts, in the peripheral blood give rise to the classic features of acute leukemias, characterized by anemia, leukocytosis and/or leukopenia, and thrombocytopenia with associated homeostatic and coagulation defects. Leukemias are classified based on the degree of differentiation of the involved cells, the predominant cell type, and the more or less rapid course of the disease. According to their course, leukemias are distinguished as acute or chronic. Specifically, acute forms, with sudden onset and aggressive course, are characterized by monomorphic and undifferentiated cell lines (blasts) that infiltrate hematopoietic tissues. Chronic forms, on the other hand, are marked by the proliferation of a more differentiated and mature hematopoietic clone, closely resembling the normal counterpart, with a slower and less aggressive course. In acute forms, it is possible to distinguish, based on the cell type they originate from, Acute Myeloid Leukemia (AML), characterized by the proliferation of blasts originating from the granulo- and monocytic lineage, and Acute Lymphoblastic Leukemia (ALL), characterized by the proliferation of blasts originating from the B or T lymphoid lineage [3].

1.3. Acute lymphoblastic leukemia in childhood

ALL is the most frequent neoplasm in the pediatric age group, accounting for over a third of all childhood cancers and approximately 80% of all cases of acute leukemia. In adults, however, ALL represents only 20% of acute leukemias [3]. The etiology of ALL is still unknown, but certain predisposing conditions such as Fanconi syndrome, Bloom syndrome, and Down syndrome can increase the risk of developing this disease. This risk is also elevated in individuals exposed to chemicals, ionizing radiation, chemotherapy drugs, and viral infections [4]. In Western countries, about 85% of ALL cases are due to the expansion of a clone of precursor B-cell (pB-ALL), while 15% correspond to aberrant T-cell precursors (T-ALL).

Over the years, various classification criteria have been proposed for ALL based on cytomorphological features (Figure 3).

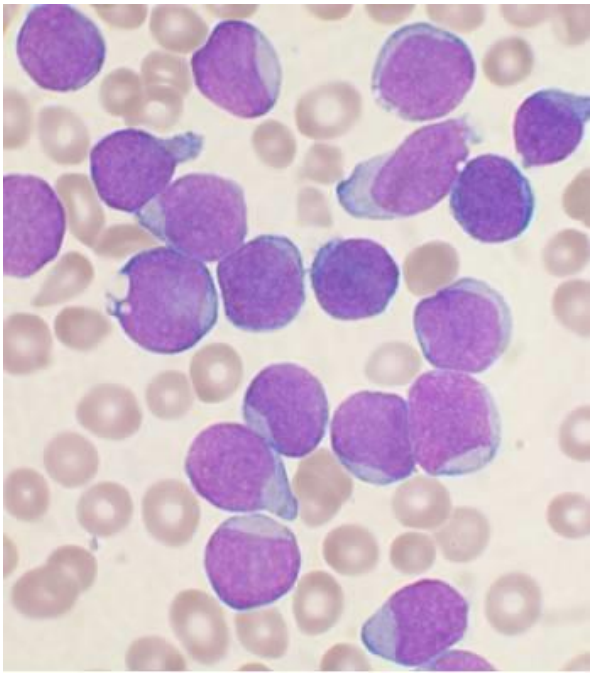


Figure 3: Peripheral blood smear of Acute Lymphoblastic Leukemia stained with Wright-Giemsa.

The French-American-British (FAB) classification system has categorized Acute Lymphoblastic Leukemia (ALL) into three primary groups based on nuclear-cytoplasmic characteristics:

- L1: Comprising uniformly small blasts with limited cytoplasm, predominantly observed in children, with an incidence ranging from 10-25%.

- L2: Distinguished by larger blast cells of variable sizes, irregular nuclear contours, and prominent nucleoli. This subtype is more prevalent in adults, with an incidence of 70-80%.

- L3: In this variant, blasts are generally large, relatively uniform, and exhibit intensely basophilic vacuolated cytoplasm. It is more common in children, with an incidence of 1-5% [5].

In contrast to other types of leukemia, the morpho-cytochemical diagnostics do not provide sufficient discrimination of subtypes in the characterization of ALL. Therefore, the application of immunological techniques is necessary for a complete diagnostic definition. In particular, the following subgroups of ALL can be identified:

1.	B lineage ALL[a] (CD19+ and/or CD79a+ and/or CD22+):	
	pro-B-ALL (B-I) (no expression of other differentiation B-cell antigens)	
	common all (B-II)	CD10+
	pre-B-ALL (B-III)	Cytoplasmic IgM+
	mature B-ALL (B-IV)	Cytoplasmic or surface kappa or lambda+
2.	T lineage ALL[b] (cytoplasmic/membrane CD3+):	
	pro-T-ALL (T-I)	CD7+
	pre-T-ALL (T-II)	CD2+ and/or CD5+ and/or CD8+
	cortical-T-ALL (T-III)	CD1a+
	mature-T-ALL (T-IV)	membrane CD3+, CD1a−
	α/β + T-ALL (group a)	anti-TCR α/β+
	γ/δ + T-ALL (group b)	anti-TCR γ/δ+
3.	ALL with myeloid antigen expression (My + ALL) (see text)	

[a]Positive at least with two of the three markers. Most cases are TdT+, HLA-DR+ except B-IV which often is TdT−.
[b]Most cases are TdT+, HLA-DR−, CD34− but these markers are not considered for diagnosis or disease classification

Table1:Classification of Acute Limphoblastic Leukemia (ALL) .

1.4. Epidemiology

Epidemiology Acute lymphoblastic leukaemia, like cancer in general, probably arises from interactions between exogenous or endogenous exposures, genetic (inherited) susceptibility, and chance (figure 1). These factors account for the roughly 1 in 2000 risk of the disease in childhood (0–15 years). The challenge is to identify the relevant exposures and inherited genetic variants and decipher how and when these factors contribute to the multistep natural history of acute lymphoblastic leukaemia from initiation (usually in utero) through the largely covert evolution to overt disease.[8] The rarity of the illness and the existence of biologically distinct subtypes that might not share common causative mechanisms complicates. For example, in infants acute lymphoblastic matters[12], leukaemia is usually associated with MLL rearrangement, and the remarkably high concordance rate in monozygotic twins (approaching 100% in those with a single or mono chorionic placenta) suggests that leukaemogenesis is largely complete at birth.[9] By contrast, incidence of non MLL-rearranged B lymphoblastic leukaemia peaks between 2 and 5 years and has a concordance rate of 10–15%, suggesting that, although initiation in utero usually occurs, other so-called promotional exposures are probably necessary for dis ease emergence.[9]

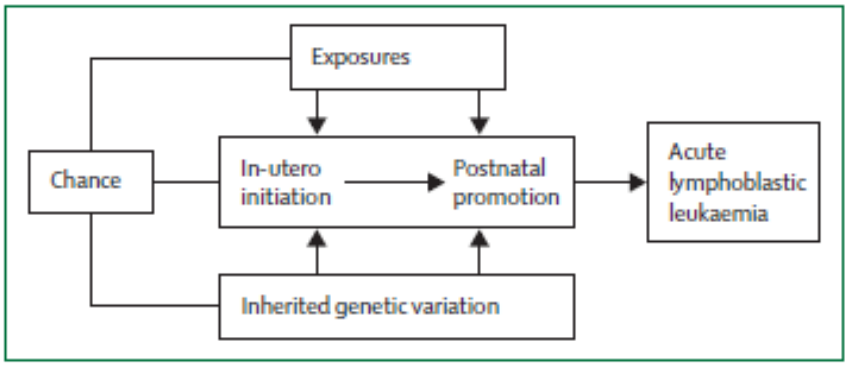


Figure 4: Composite causality of childhood acute lymphoblastic leukemia. Exogenous (eg, infection) and endogenous (eg, inflammation, oxidative stress) exposures, normal allelic variation in inherited genes, and chance all have roles in the covert natural history of childhood acute lymphoblastic leukemia. Cancer causation is closely linked with chance—eg, incidental external exposure, damage to a relevant oncogene in a relevant cell (stem or progenitor cell), chance events at conception implicating parental gene shuffling and recombination.

1.5. Genetic basis

High-resolution profiling of genetic alterations has transformed understanding of the genetic basis of acute lymphoblastic leukaemia. That most childhood cases harbour gross chromosomal alterations has been known for several decades.[36] In B-cell disease, these alterations include high hyperdiploidy with non-random gain of at least five chromosomes (including X, 4, 6, 10, 14, 17, 18, and 21); hypodiploidy with fewer than 44 chromosomes; and recurring translocations including t(12;21)(p13;q22) encoding ETV6–RUNX1, t(1;19)(q23;p13) encoding TCF3–PBX1, t(9;22)(q34;q11) encoding BCR–ABL1, rearrangement of MLL at 11q23 with a wide range of partner genes, and rearrangement of MYC into antigen receptor gene loci. Dysregulation of TAL1, TLX1, TLX3, and LYL1, particularly by rearrangement into T-cell antigen receptor loci, often occurs in T lymphoblastic leukaemia. These changes are of key importance in both pathogenesis and clinical management (figure 5). Many chromosomal rearrangements disrupt genes that regulate normal haemopoiesis and lymphoid development (eg, RUNX1, ETV6), activate oncogenes (eg, MYC), or constitutively activate tyrosine kinases (eg, ABL1).

Several are significantly associated with outcomes, particularly in B-cell disease, and are used in risk stratification. High hyperdiploidy and ETV6–RUNX1 rearrangement are associated with favourable outcome, whereas low hypodiploidy and MLL rearrangement (especially in infants and adults) are associated with poor prognosis in both children and adults. However, many of these alterations alone do not induce leukaemia in experimental models, and no chromosomal alteration is noted in many cases, suggesting that additional submicroscopic genetic alterations contribute to leukaemogenesis. High-resolution microarray profiling of DNA copy number alterations (deletions and gains) and sequencing have led to identification of several novel structural genetic alterations and sequence mutations (some of which are being investigated as prognostic markers or therapeutic targets) that defi ne new subtypes of disease, contribute to leukaemogenesis, and affect treatment responsiveness.[37–42] Importantly, many sub types of acute lymphoblastic leukaemia are

characterized by distinct constellations of structural genetic alterations that together drive establishment of the leukaemic clone.

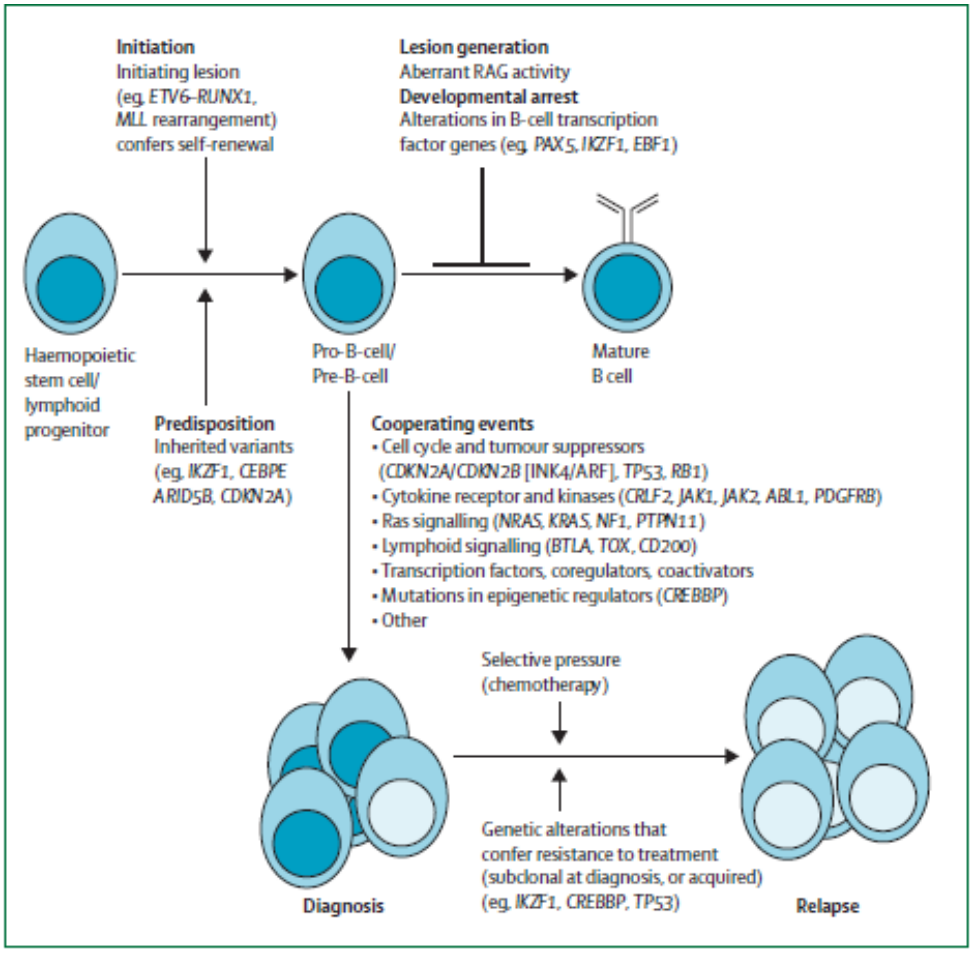


Figure 5. **Genetic pathogenesis of B lymphoblastic leukemia at diagnosis and relapse.**

1.6 Recurrent Genetic Aberrations in ALL

The most frequent genetic alteration (25%) in pediatric pB-ALL is the translocation t(12;21)(p13;q22) that generates the ETV6-RUNX1 fusion gene. Rearrangements of the MLL gene, especially the t(4;11)(q21;q23) translocation, giving rise to the MLL-AF4 fusion gene, represent key events in the development of B-cell ALL in infants under one year (pBLLA infant). However, the t(9;22)(q34;q11) translocation, which results in the formation of the BCR-ABL1 fusion gene, encoding a constitutively active chimeric tyrosine kinase protein, characteristic in B-ALL. It is present in approximately 40% of adult pB-ALL cases and around 5% of pediatric cases [10]. The t(1;19) mutation causing the fusion of the E2A-PBX1 genes is common in 5% of ALL and 20% in pre-B ALL and is associated with a favorable prognosis when intensive therapeutic protocols are used. Not only there are structural chromosomal abnormalities underlying the development of pB-ALL, but also recurrent numerical abnormalities of various types, which are predictive of patient outcomes. Hyperdiploidy (particularly the high-hyperdiploidy condition, > 50 chromosomes) is a particularly significant alteration in the context of pB-LLA. It is present in 30% of pediatric cases and slightly over 9% of adults. In this type of alteration, the main chromosomes involved include the following: 4, 6, 10, 14, 17, 18, 21, and X. Prognosis varies depending on the number of excess chromosomes and which chromosomes are involved. Hypodiploidy (≤ 45 chromosomes), on the other hand, has (J. Zhang et al., *Germline Mutations in Predisposition Genes in Pediatric Cancer.* N Engl J Med 2015) a very low frequency (1-2%) in pB-ALL, and in this case, prognosis depends on the type and number of chromosomes lost [11]. Overall, all these genotypes are found in only 60% of pB-ALL cases, while the genetic abnormalities in the remaining 40% are currently being identified and are rare. Approximately 40% of pB-ALL patients have deletions, amplifications, point mutations, or structural rearrangements in genes encoding various regulators of normal lymphocyte development, including E2A, EBF1, IKZF1, BTG1, ETV6, and RB1 [12-14]. In 35% of cases, mutations have been found in FLT3 (associated with MLL gene rearrangements), PTPN11, and RAS (associated with

hyperdiploidy). Finally, 15-20% of pB-ALL patients negative for recurrent rearrangements have a gene expression profile similar to BCR-ABL-positive leukemia and an unfavorable outcome, leading to their classification as BCR-ABL-like ALL [15].

1.7 Therapeutic Approaches for ALL

In recent years, there has been significant therapeutic improvement in the treatment of ALL, particularly in the development of more effective chemotherapy regimens and appropriate supportive care. In children, the cure rate has reached approximately 80%, while in adults, despite progress, results remain relatively less successful, with a success rate of about 40% [1]. Treatment choices are based on prognostic factors, and patients are stratified according to disease characteristics into risk classes, each corresponding to a specific treatment protocol. In general, patients undergo various phases of treatment, including the so-called induction therapy, consolidation, re-induction, and maintenance phases [3,13].

Induction therapy aims to achieve complete remission, eradicating more than 99% of the initial leukemic cells and restoring normal hematopoiesis. It is divided into three phases:

- The pre-phase, common to all patients, involves the administration of Prednisone from day 1 to day 7. The response to this drug after 7 days of treatment (the end of the pre-phase) is used as a stratification criterion to assess treatment effectiveness in the initial phases.
- Phase Ia (from day 8 to day 33) and phase Ib (from day 33 to day 78) involve cytostatic therapy using a combination of drugs (glucocorticoids + vincristine + anthracycline or asparaginase).

Once normal hematopoiesis is restored, patients in remission become candidates for consolidation or re-induction therapy to minimize the development of resistant cells and control minimal residual disease. This therapeutic phase follows two different modes, consolidation or re-induction, depending on the patient's risk group. For patients with a poor initial response to treatment, allogeneic hematopoietic stem cell transplantation is the primary post-induction therapeutic option. As for BCR-ABL-positive patients with the t(9;22) translocation, a targeted therapeutic protocol has

been developed in recent years, involving the administration of the drug Gleevec (Imatinib), a selective inhibitor of ABL tyrosine kinase activity. Maintenance therapy in ALL patients is extended (12-18 months) and involves the combined administration of 6-mercaptopurine and methotrexate to halt leukemia growth and continue cytoreduction until complete elimination of leukemic cells. The mode of administration for maintenance therapy is the same for all risk classes [14, 16,17].

1.8 Prognostic Factors in Pediatric ALL

A rigorous assessment of relapse risk at diagnosis is necessary for targeted therapy. It is essential to evaluate the patient's age, leukocyte count, genotype of leukemic cells (especially known genetic translocations with prognostic significance), and the patient's response to the induction therapy [5].

1.9 Clinical Characteristics

Age at diagnosis and leukocyte count are significant prognostic indicators in B-cell ALL but not in T-cell ALL. An age between one and nine years and a leukocyte count of less than $50x10^{\wedge 9}$ cells per liter define standard-risk B-cell leukemia. Patients under one year of age are considered to have an unfavorable prognosis. For adults (>15 years), in general, the outcome worse with increasing age and leukocyte count. However, there are no precise guidelines for stratification based on these criteria [17].

1.10 Genotype of Leukemic Cells

The different clinical outcomes associated with different subtypes of ALL can be attributed to the sensitivity or resistance of leukemic cells that have specific genetic alterations to drugs [18]. In pB-ALL, hyperdiploidy, the t(12;21) translocation, and trisomies of chromosomes 4, 10, and 17 are associated with a favorable prognosis, while hypodiploidy, and the t(4;11) and t(9;22) translocations indicate an unfavorable prognosis. The mechanisms by which these genetic alterations induce different aggressiveness and drug sensitivity are still unknown and subject to study. For example, in

the case of hyperdiploid ALL, leukemic cells are more prone to undergo apoptotic processes due to the accumulation of methotrexate polyglutamates, making them more sensitive to methotrexate and mercaptopurine treatment [15]. Additionally, cooperative deletions in genes encoding various regulators of normal lymphocyte development, such as E2A, EBF1, IKZF1, BTG1, ETV6, and RB1, are particularly common in patients with an unfavorable prognosis [9,19].

One possible reason for inadequate therapeutic response, based on the biological characteristics of leukemic cells, may be the presence of polymorphisms in genes encoding drug-metabolizing enzymes, transporters, receptors, and pharmacological targets. These polymorphisms can cause variations in drug absorption, distribution, metabolism, and clearance processes, resulting in varying responses to therapy and toxicity among different patients [17,20].

1.11 Response to Pharmacological Treatment

Since the 1980s, the importance of measuring treatment efficacy in vivo has been demonstrated. This parameter has the most significant prognostic value compared to any other clinical or biological criteria considered so far [17,21].

- Morphological analysis on day 33: Initially, therapy response was assessed solely through morphological analysis of bone marrow aspirate after 33 days of induction therapy. Bone marrow is considered in morphological remission if it contains less than 5% blast cells among nucleated cells [20].

- Response to Prednisone: In the early 1990s, the evaluation of treatment response in the initial phases was added as a risk factor through cytomorphological analysis of peripheral blood after 7 days of Prednisone treatment. Although it is an important and independent clinical parameter, it has low sensitivity [23], as it can only discriminate a limited number of high-risk patients. Additionally, morphological analysis can be challenging and misleading [20].

- Minimal Residual Disease (MRD): In recent years, the measurement of minimal residual disease using molecular biology techniques capable of detecting extremely low levels of blast cells (sensitivity of 10^{-4}-10^{-5}, meaning one leukemic cell per 10^{4}-10^{5} normal leukocytes) has been

introduced as a stratification criteria. It has been observed that MRD evaluation correlates with key stratification parameters and is also an independent prognostic factor that enhances the accuracy of patient classification into different risk groups [20,21,24]. A reliable technique to identify minimal residual disease must possess the following requirements:

- Sensitivity of at least 10^{-4};
- Specificity in distinguishing between malignant and normal cells to prevent false-positive results;
- Quantifiability within a wide range;
- Long-term stability in detecting leukemic markers to prevent false negatives, especially in long- term studies;
- Reproducibility across different laboratories;
- Easy standardization;
-Rapid availability of results.

Chapter 2. DNA repair machinery

2.1 The DNA Repair System and the Fanconi Pathway

During an individual's lifetime, the genome sustains double-strand DNA damage from three primary sources: environmental agents (including ultraviolet (UV) radiation from sunlight, ionizing radiation, and chemical substances); reactive oxygen species generated during regular oxidative respiration, and intrinsic factors related to DNA due to the labile nature of the chemical bonds within the molecule that undergo spontaneous hydrolysis under physiological conditions, resulting in the loss of purines (depurination) or amino groups (deamination). Therefore, various mechanisms have evolved to repair DNA breaks and prevent mutations. One such mechanism is "Non-homologous end joining (NHEJ)" for repairing double-strand breaks (DSBs).

In the network of DNA damage response, it is now widely accepted that Fanconi anemia (FA) proteins are involved, playing a crucial role, particularly in the homologous recombination mechanism following double-strand DNA breaks.

1. "Non-homologous end joining (NHEJ)," in which damaged DNA has not yet been replicated to provide a homologous sequence template. The free ends of the double strand are joined through the NHEJ pathway, often resulting in the loss of sequence information, and the repair process proceeds without a template.

2. The second mechanism is homologous recombination (HR), dependent on DNA replication to provide a homologous sequence to the damaged strand. Cells progressing through the S phase can repair broken chromosomes using the sequence information from the sister chromatid as a template.

3. The third mechanism is nucleotide excision repair (NER), which repairs damage involving strands ranging from 2 to 30 nucleotides. In NER, the lesion is recognized and excised, followed by DNA synthesis, repairing the region using the complementary strand as a template.

4. The fourth mechanism is base excision repair (BER), evolved for the repair of point mutations. The enzymatic process begins with the activation of a DNA glycosylase that recognizes the altered base and breaks the N-glycosidic bond. An AP-endonuclease 1 (AP: apurinic or apyrimidinic site, generally known as an abasic site) then removes the nitrogenous base, leaving the phosphate and deoxyribose.Subsequently, a lyase removes the phosphate and sugar, allowing a DNA polymerase to incorporate the new nucleotide, with ligase sealing it into the strand. BER can repair the deamination of cytosine into uracil or the transformation of guanine into 8-oxo-guanine, an adenine analogue.

From a functional perspective, the sixteen identified FA genes can be traditionally categorized into three subgroups:

1. The proteins FANC-A, -B, -C, -E, -F, -G, -L, and -M constitute the first group. These proteins assemble into a multiprotein complex called the "core complex" or more simply, complex I, stabilized by weak interactions between different sub-complexes. The function of this complex, along with two "FA-interacting proteins" known as FAAP24 and FAAP100, is to control the monoubiquitination of FANCD2 and FANCI, a process essential for DNA repair system activation. Mutations affecting even a single subunit render cells incapable of ubiquitinating FANCD2 and FANCI, as the stability of the entire FA complex is compromised.

2. The second group consists of FANCD2 and FANCI, which interact with each other in a dynamic complex (complex II) that moves in and out of chromatin based on post-translational modifications. When activated through ubiquitination, the complex localizes to nuclear foci induced by DNA damage. These two proteins, similar in size and structure, are interdependent for monoubiquitination. In cells lacking FANCD2, FANCI remains unubiquitinated.

3. The third group (complex III) comprises the proteins FANCD1/BRCA2, FANCN (or PALB2, a partner and co-localizer of BRCA2), FANCJ (or BRIP1, interacting with BRCA1), FANCO (or RAD51C), FANCP (or SLX4), and FANCQ (or ERCC4, excision repair cross-complementing rodent repair deficiency group 4). Cells with defects in any of these genes exhibit normal levels of

ANCD2 monoubiquitination, indicating that the corresponding proteins function downstream of complex II or in a parallel pathway [34]. The proteins FANCD1, FANCJ, FANCO, and FANCQ are closely associated with recombination mechanisms. Recombination between the template and the defective molecule allows the formation of two error-free intact DNA molecules.

As for FANCN, it appears to be important for recruiting and accumulating FANCD1 at the repair site, while FANCP acts as support for various endonucleases working in concert to resolve any secondary structures formed during repair events [35].

The activation of the FA-BRCA pathway depends, at least in part, on ATR (ataxia telangiectasia and Rad3-related protein) and CHK1 (checkpoint kinase 1), two kinases activated in turn by replicative stress. Many FA proteins, such as FANCM, FANCA, FANCE, and FANCG, undergo phosphorylation after DNA damage. These proteins may act as signal transducers through which ATR signals the presence of replicative problems to the cell [36]. After phosphorylation events and interactions between different FA proteins, conformational changes result in the exposure of nuclear localization domains and subsequent entry into the nucleus (Figure 6). An essential activation element is provided by FANCM, which, despite possessing a helicase domain, functions more as an ATP-dependent translocase, recognizing and stabilizing the stalled replication fork, and recruiting the core complex to the site [36]. At this stage, the monoubiquitination of FANCD2 and FANCI can occur, a key event in the FA pathway. This is a post-translational modification event that requires the formation of a covalent bond between glycine 76 of ubiquitin and the ε-amino group of a lysine on the target molecule [38]. This mechanism is observed in numerous cellular processes that require changes in subcellular localization or the activity of certain components. The eight FA proteins in group I, FAAP24, FAAP100, and UBE2T (ubiquitin-conjugating enzyme, E2), when assembled into a complex, are essential for binding the ubiquitin molecule to Lys 561 and Lys 523 of FANCD2 and FANCI, respectively [39]. The stability of the complex is severely compromised when one of the subunits is not produced or is defective; in both cases, the absence of any of these components compromises the modification of FANCD2 and FANCI. Monoubiquitination of FANCI does not appear to play a direct role in the FA pathway; however, it acts as a kind of molecular switch to

activate the FA pathway [40]. The ubiquitination reaction is particularly associated with the presence of FANCL, one of the complex subunits, which would serve as the E3 ligase as it is the only one with ubiquitin ligase activity due to its WD40 and PHD (plant homeodomain) domains that allow interaction with the E2 enzyme, UBE2T. Monoubiquitination is a reversible and regulated process: the specific deubiquitinating enzyme for this pathway is ubiquitin-specific protease-1 (USP1). It would act as a negative regulator of the FA pathway by removing ubiquitin from FANCD2 during the G2 phase of the cell cycle [41] (Figure 6). Following monoubiquitination, FANCD2 and FANCI, utilizing the translocase activity of FANCM, migrate to nuclear foci near the DNA lesion site, where they can coordinate repair activities along with the FA proteins from complex III and numerous other proteins.

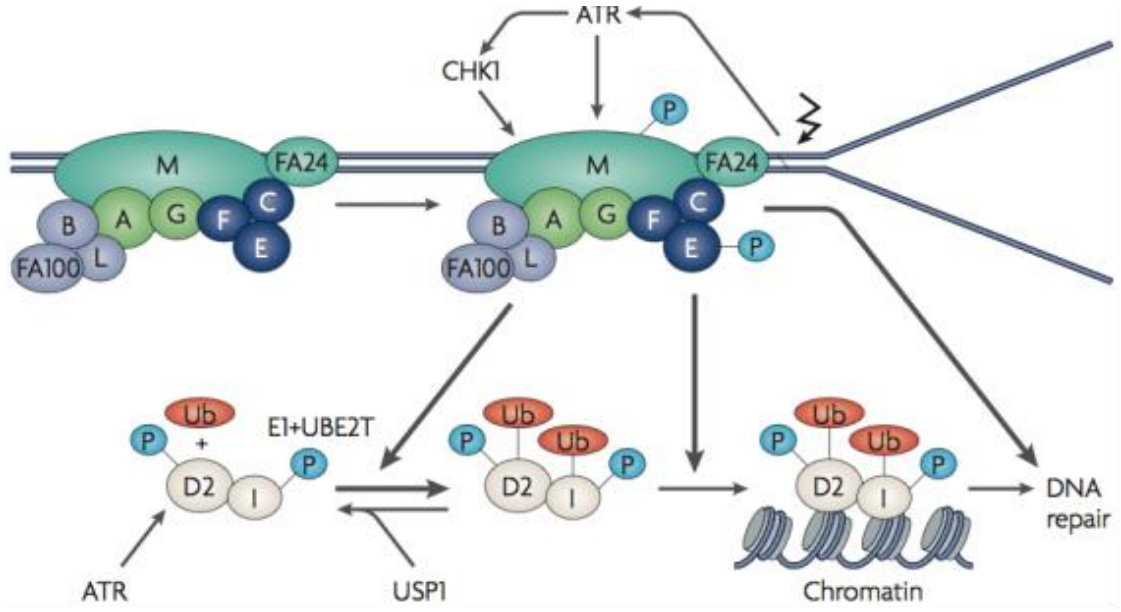


Figure 6: Signalling through the Fanconi anaemia (FA)–breast cancer susceptibility (BRCA) network in response to DNA damage.

A fundamental characteristic of the FA pathway is that its activity is regulated by the ubiquitination state of FANCD2 and is inducible only in the case of DNA damage. It is of significant importance to emphasize that the pathway's activity is limited to the S phase of the cell cycle. However, it can be induced in response to DNA-damaging agents such as ionizing radiation, DEB, MMC, hydroxyurea, etc. [42]. Such regulation may occur by affecting the presence or removal of ubiquitin from FANCD2, the assembly of the nuclear FA complex, or the presence or absence of the complex at the damaged site [43].

2.2 The DNA Damage Repair Pathway: Complex III

Following ubiquitination, in proximity to nuclear foci, FANCD2-Ub acts as an anchoring site for certain nucleases such as FAN1 (FA-associated nuclease 1), FANCP/SLX4, SLX4-associated, MUS81-EME1, and XPF-ERCC1. The role of these nucleases is to promote the release of the replication fork by cleaving near the cross-link between the two DNA strands. The result is a double-strand break resolved through the use of a specific low-fidelity polymerase via the translesion synthesis (TLS) mechanism [44]. When the fork is not blocked, replication can continue, and the double-strand break will be repaired later through homologous recombination and nucleotide excision repair (NER) [35] (Figure 7).

The other FA proteins of group III, namely FANCD1, FANCJ, FANCN, and FANCO, play a significant role in the homologous recombination process. They facilitate progression through various HR intermediates and enhance the accumulation of key repair proteins at the foci, such as RAD51, BRCA1, BLM, TLS polymerase, RPA, and ATR. The final phase of the FA pathway, the activation of specific repair processes, begins with the deubiquitination of FANCD2 by USP1. This event likely removes FANCD2 from the damage site, allowing the FANCD1/BRCA2 and FANCN complexes to interact with RAD51, the key protein in homologous recombination, activating it [40].

The last element of the FA pathway, whose action occurs downstream of monoubiquitination, is FANCJ. This protein is a DNA-dependent ATPase with a 5'-3' helicase function, which directly

binds to the BRCT domains of BRCA1, connecting the FA pathway with another DNA repair pathway that is still not well understood but is believed to have analogies with TLS [44]. Additionally, FANCJ appears to be capable of removing DNA structures that hinder lesion repair [40].

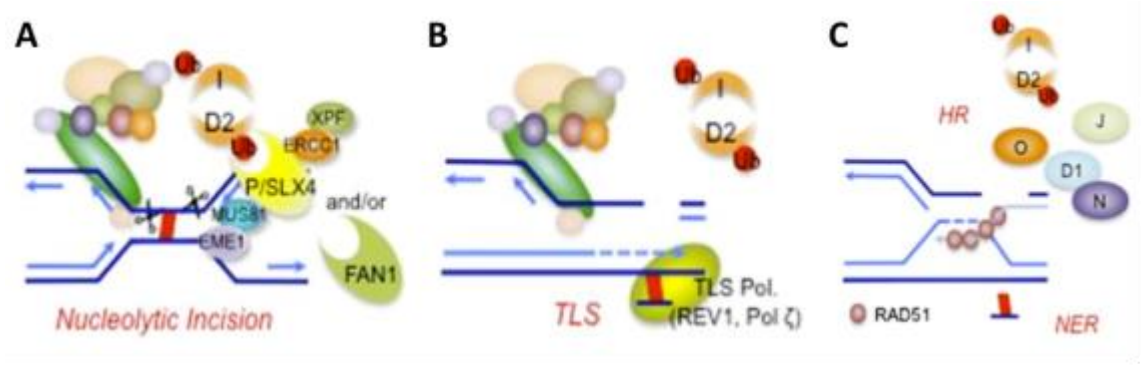


Figure 7. Interaction of the 15 FA proteins in a common ICL repair pathway in S phase. (A) Two replication forks converge on the DNA ICL that covalently links the two strands of DNA. (B) The FANCM–FAAP24–MHF1/2 complex recognizes the stalled replication fork structure and recruits the FA core complex to the ICL region. The translocase activity of FANCM prevents the collapse of replication fork independent of FA pathway activation. FANCM also initiates ATR-CHK1-dependent checkpoint response, which in turn phosphorylates multiple FA proteins, including FANCA/E/D2/I. (C) The FA core complex, a ubiquitin E3 ligase, monoubiquitinates FANCD2 and FANCI, and the ID heterodimeric complex is recruited to the DNA lesion. (D) FANCD2-Ub acts as a platform to recruit multiple nucleases to coordinate nucleolytic incisions flanking the ICL. FANCP/SLX4, which interacts with ERCC1–XPF and MUS81–EME1 structure-specific nucleases, and FAN1 5′-flap endonuclease are good candidates for this process. Both SLX4 and FAN1 contain the UBZ4 UBM essential for FANCD2-Ub-dependent recruitment to the DNA lesion. (E) Unhooking leaves cross-linked nucleotides tethered to the complementary strand, which is bypassed by TLS, mediated by specialized TLS polymerases such as REV1 and Pol ζ. (F) Incision creates a DSB, which is repaired by HR. Downstream FA proteins promote RAD51-dependent strand invasion and the resolution of recombinant intermediates. NER removes remaining adducts and fills the gap. (G) The USP1–UAF1 DUB complex removes monoubiquitin from FANCD2-I and completes the repair.

Today, it is known that the FA pathway constitutes only a part of the complex network of mechanisms that control genome integrity. This is demonstrated by the discovery of a wide range of interactions between FA proteins and other components involved in DNA repair, such as the MRN complex (MRE11, Rad51, and NBS1), BRCA1 and BRCA2 proteins, ATR, ATM, and BLM. Mutations in some of these proteins cause other DNA repair diseases [44], such as ataxia telangiectasia, Bloom syndrome, and Fanconi anemia, which exhibit chromosomal instability, predisposition to leukemia, and hypersensitivity to agents like ultraviolet radiation, X-rays, and alkylating substances. Moreover, the hematopoietic tissue appears to be one of the most sensitive to DNA damage caused by radiation or chemotherapy, which is why diseases like ataxia telangiectasia, despite presenting genomic instability issues with an increased risk of developing tumors do not have the same incidence of bone marrow failure as seen in Fanconi anemia, despite presenting genomic instability. Therefore, it seems possible that FA proteins play a critical role in maintaining hematopoietic stem cells: the initial decline in their functionality may originate due to telomeric stability defects and the high rate of cell apoptosis in FA. Subsequently, selective pressures may promote the development of mutant cellular clones (facilitated by genomic instability), leading to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) [45].

2.3 BRCA1 and BRCA2 Genes

BRCA1 and BRCA2 (Breast Cancer susceptibility gene 1 and 2) encode high molecular weight proteins with similar expression and subcellular localization patterns. They are both expressed in various tissues, primarily during the G1/S phase of the cell cycle, indicating their role in DNA replication. Additionally, BRCA proteins are located in the nuclei of somatic cells, where they coexist in distinctive subnuclear foci that redistribute based on the location of DNA damage.

The BRCA1 gene, mapped to the long arm of chromosome 17 (17q21), was discovered by Mary-Claire King in 1994 [46]. This gene consists of 24 exons, with a total length of 5693 base pairs. Of these exons, 22 encode a 1863-amino acid protein (220 kDa), while exons 1 and 4 are processed during transcription. The BRCA2 gene, discovered in 1995 and mapped to the long arm of chromosome 13 (13q12), comprises 27 exons (11385 nucleotides) and encodes a nuclear protein of 3418 amino acids (384 kDa).

BRCA1 and BRCA2 are considered tumor suppressor genes as they are involved in DNA repair through homologous recombination (HR), in transcription regulation and cell cycle control. They perform upstream control, which involves their roles in DNA repair systems, and downstream regulation, which is involved in controlling the cell cycle through various checkpoints [47].

Regarding homologous recombination, the literature suggests that BRCA1 and BRCA2 have different roles, even though they are located within a single macromolecular complex. It has been demonstrated that while BRCA1 plays a more general role, acting as a mediator between DNA damage signals and repair effectors, BRCA2 binds to and controls the activity of the RAD51 protein.

a) ***Role of BRCA1***

As a consequence of a double-stranded DNA break, the damaged site is immediately marked through the phosphorylation of histone H2A-X. BRCA1 is phosphorylated and consequently activated. In its

active form, it migrates to DNA replication forks, where it associates with repair-specific protein complexes [48]. Phosphorylation occurs at various serine residues (Ser1387, Ser1457, Ser988, Ser1423, Ser1524) by ATM, ATR (ATM-related kinase), and CHK2 kinases [52,53]. BRCA1 is part of many macromolecular complexes (figure 8). The first of these is the MRE11/RAD50/NBS1 complex, also known as the MRN complex. The first step in DNA repair is the formation of a 3'-overhanging single-stranded DNA. The MRE11 nuclease generates stretches of ssDNA, which are then bound by RAD51. By inhibiting the nuclease activity of MRE11, BRCA1 can regulate the length and quantity of ssDNA stretches that form, thus serving as a coordinator of the genetic damage response [17]. BRCA1 also interacts with the SWI/SWF complex, responsible for chromatin remodeling, which allows for easier access to damaged DNA by repair factors.

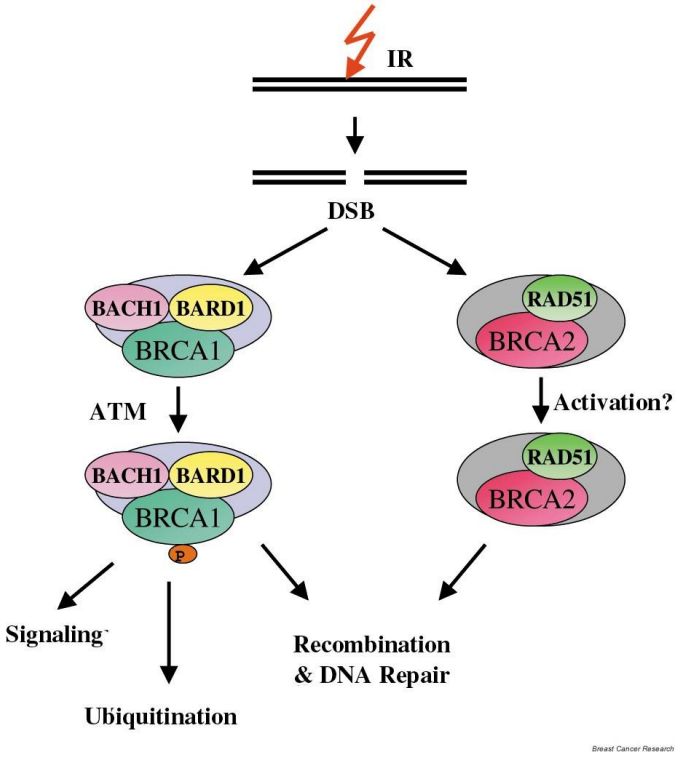


Figure 8. Distinct complexes formed by BRCA1 and BRCA2 are involved in a variety of cellular processes such as damage signalling, protein degradation and both direct and indirect effects on the mechanisms of homologous recombination and DNA repair. Defects in these processes can lead to inefficient repair of DNA damage, genomic instability and tumourigenesis.

b) ***Role of BRCA2***

BRCA2's role in DNA repair is more direct, as it directly binds RAD51 at the BRCT domains and the carboxy-terminal tail. RAD51 is the eukaryotic homolog of the bacterial RecA protein, which has catalytic activity critical for homologous recombination. RAD51 can bind to single-stranded DNA (ssDNA), forming a nucleoprotein filament that pairs with its homologous region in the double-stranded DNA of the sister chromatid and promotes strand exchange [49,50]. A proposed model for the in vivo functioning of the BRCA2-RAD51 complex involves an inactive state where RAD51 cannot bind to DNA and an active state in which RAD51 performs recombination [51].

The BRC domain of BRCA2 can block the formation of nucleoprotein filaments and can cleave already formed ones. Maintaining the inactive state is necessary to prevent undesired activation of recombination during DNA replication; conversely, the cleavage between BRCA2 and RAD51 is essential for the repair process. The transition from the inactive state to the active state (and vice versa) is not due to the cleavage of the bond between BRCA2 and RAD51 but rather results from post-translational modifications such as phosphorylation/dephosphorylation of one or both proteins in response to DNA damage.

In the work of *Antonio Valeri et al.* [54], ectopic expression of BRCA1 was observed in BCR-ABL positive hematopoietic cells, interfering with the translocation of FANCD2 to damaged DNA sites, compromising the genomic stability of cells. Meanwhile, in the study by *Zhang et al*. [55], BRCA2 is among the most commonly mutated genes in young patients with tumors under the age of 20.

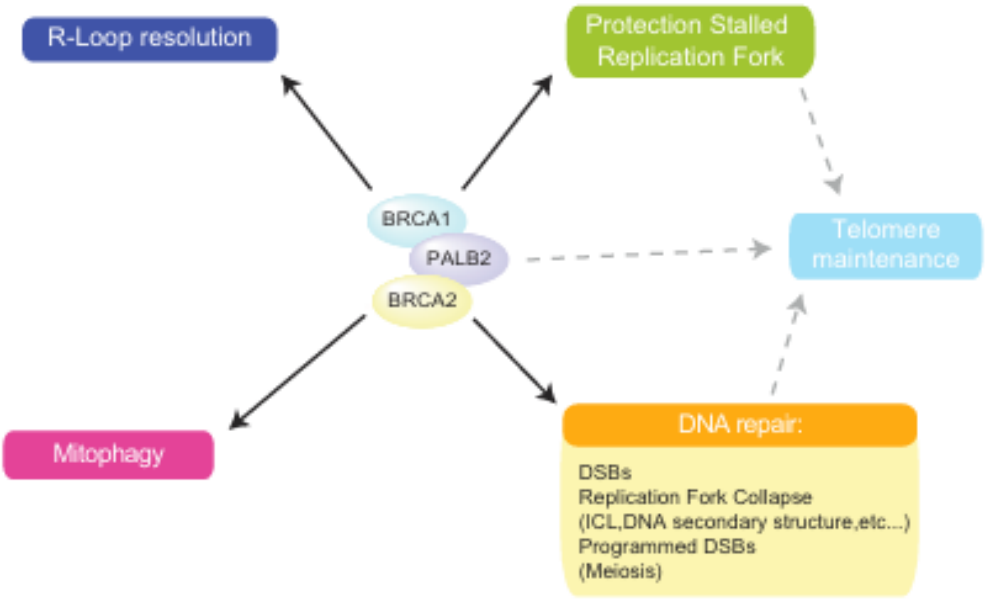


Figure 9. Stalled Replication Fork BRCA1 PALB2 BRCA2 Telomere maintenance DNA repair: DSBs Replication Fork Collapse (ICL,DNA secondary structure,etc...) Programmed DSBs (Meiosis) BRCA2 functions in the maintenance of genome stability. Bound to BRCA1 and PALB2, BRCA2 participates in multiple biological processes that are critical to maintain genome stability. First, BRCA2 is a key player in the repair of DNA lesions including DNA double-strand breaks (DSBs) and intrastrand crosslinks (ICLs). Moreover, BRCA2 has a DNA repair independent function: it prevents nucleolytic degradation at stalled replication forks. Both of these functions are directly or indirectly involved in the maintenance of telomeres. BRCA2 is required for the processing of R-loops in collaboration with the TREX-2 complex. More recently, BRCA2 has been involved in mitophagy and the clearance of damaged mitochondria, thereby indirectly preserving genome stability.

2.4 The NBS1 Gene

The NBS1 gene consists of 16 exons and is located on chromosome 8q21. It encodes a 754-amino acid protein with a molecular weight of 85 kDa called nibrin (p95). Nibrin is involved in the double-strand DNA break repair complex by forming the MRN complex (MRE11/RAD50/NBS1). In cultured cells exposed to ionizing radiation, the MRN complex forms nuclear foci at sites where DNA repair occurs [56]. In response to double-strand DNA damage, ATM phosphorylates NBS1 on serine 343, which, in turn, promotes the phosphorylation of the FANCD2 protein on serine 222. This way, NBS1 protein binds to other components of the MRN complex and co-localizes with the activated form of FANCD2 in subnuclear foci where DNA repair via homologous recombination is required [57].

Biallelic mutations in the NBS1 gene cause Nijmegen Breakage Syndrome, characterized by short stature, microcephaly, recurrent infections, and an increased risk of lymphoma and leukemia [58]. Additionally, an increased risk of acute lymphoblastic leukemia (ALL) has been observed in individuals with the rs1805794 C/G polymorphism in NBS1, as it appears to be a genetic modification that promotes the development of leukemia [59].

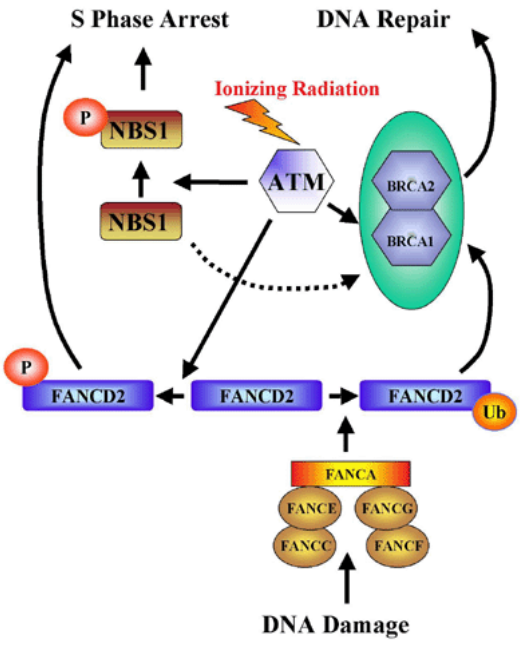


Figure 10. *The Fanconi anaemia/BRCA pathway*. D'Andrea,A.D. and Grompe,M. (2003). Nat. Rev.Cancer

Chapter 3. Role of the DNA Repair Machinery in Childhood B-Lineage Acute Lymphoblastic Leukemia

PROJECT HYPOTHESIS. My PhD research project we hypothesized that germline alterations and somatic disruption of the FA/BRCA pathway has recently been implicated in the pathogenesis of leukemia and may carry important implications in terms of prognosis and therapy response. Defective DNA repair pathway may not only be disease-causing, but further contribute to poor treatment outcome and poor prognosis in leukemia. The challenge is to identify molecular features of single leukemic cell patient-specific to support clinicians in choosing of the most appropriate therapeutic treatment, to avoid toxic events and preserve the advantage of the quality of therapy.

The results of this project allowed:

▪ to characterize DNA repair genes in childhood leukemia with alterations arise in pre-leukemic clone;

▪ to establish the role DNA repair genes and specific chromosomal translocations in subgroups of childhood leukemia;

▪ to show the correlation between altered DNA repair machinery and relapse and/or toxic events.

AIMS. This study is one of the projects included in the European Consortium COST-Legend on Pediatric Cancer Predisposition in ALL and NHL.

We addressed our research to specific genes as *NBS1* (Nijmegen Breakage Syndrome) and Fanconi Anemia-BRCA pathway (*BRCA1, BRCA2, FANCD2, PALB2*) involved in the DNA repair, in the attempt to identify mutations and aberrant expressions, predisposing and/or cooperating with the leukemogenic process. In order to better understand this mechanism, we aimed to study specific subgroups of childhood leukemia:

- In B-lineage acute lymphoblastic leukemia: with t(9;22) or chromosome Philadelphia positive (Ph+) (associated with a poor prognosis); with t(1;19) or E2A-PBX1 positive (associated with a good prognosis); with t(12;21) or ETV6-RUNX1 positive (good prognosis); *MLL*-rearranged positive (poor prognosis) and without known translocations defined as "B-others". In this latter group, we performed analyses of mutational status regarding *IKZF1*, *CRLF2* and *CDKN2A/B* genes, respectively, because of their well-established prognostic impact.

MATERIALS AND METHODS. The study was approved by the Local Clinical Research Ethics Committee (Catania-1). Written informed consents were obtained from the parents of each child involved in this research. We analyzed bone marrow (BM) samples from diagnosis and remission (Dx/Rem) of 111 children with B-ALL, diagnosed and treated at the Center of Pediatric Hematology Oncology, Catania Italy, from 2000 to 2017. These children were enrolled in three consecutive protocols of Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP) [AIEOP-BFM ALL 2000; AIEOP-R 2006; AIEOP BFM ALL 2009]. Biological subgroups were as follow: 16 children presenting with t(1;19); 48 with t(12;21), 9 with t(9;22)/Ph+, 5 with a KMT2A (formerly MLL) rearrangement and 33 defined as "B-Others". Traditional PCR method was used to determinate the NBS1 mutations in exons 3-6, followed by Sanger sequencing. FANCD2 and PALB2 mutations were investigated by multiplex ligation-dependent probe amplification (MLPA). Samples from healthy donors were used as wild-type controls and cells line as positive controls. A real-time PCR assay was applied to evaluate the BRCA1 and BRCA2 genes expression using the SYBR green, fluorescent dye. We used specific primers for BRCA1 exons 14-15 and BRCA2 exons 15-16, respectively. The expression of BRCA1 and BRCA2 was determined as high (H) or low (L) using the Mean and the Median of fold change (FC) as cut-off.

3.1 Sample preparation

Separation of mononuclear cells from whole blood or bone marrow aspirate.

The separation of mononuclear cells (lymphocytes, monocytes, blasts) from polymorphonuclear cells and red blood cells was performed using Ficoll gradient separation.

- The sample was slowly layered into a Falcon tube containing 3 ml of Ficoll-Paque Plus.

- Subsequently, centrifugation was carried out at 1400 rpm for 20 minutes without braking. At the end of the centrifugation, the sample was divided into multiple phases (Figure 11).

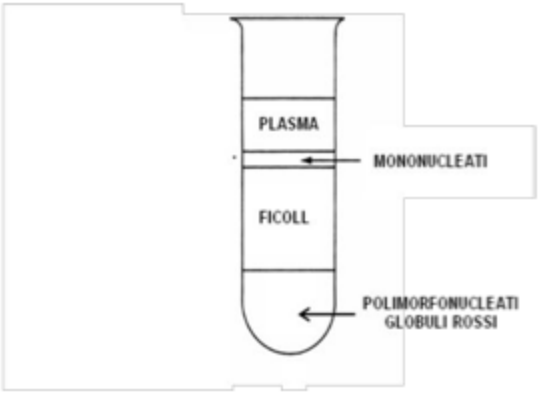


Figure 11. Division of the sample after separation using the Ficoll gradient.

- At this point, the mononuclear cells located in the ring between the plasma and the Ficoll layer were recovered using a Pasteur pipette.

- Physiological saline solution was then added to the mononuclear cells for washing, and the suspension was centrifuged at 1400 rpm for 5 minutes.

- The obtained pellet was dried and resuspended in approximately 6 mL of physiological saline solution for cell counting.

- Cell count and vitality were determined using the Trypan blue dye (which stains the cytoplasm of non-viable cells blue): 10µL of Trypan blue were mixed with 10µL of the cell suspension.

- Cell vitality was assessed under an optical microscope using a Bürker chamber (Figure 12), which allows for the measurement of the number of viable and non-viable cells per unit volume.

- The suspension was adjusted to a concentration ranging from 800,000 to 1,000,000 cells per mL by reducing the volume or diluting with physiological saline solution and then centrifuged at 1400 rpm for 5 minutes.

- The resulting cellular pellets were used for nucleic acid extraction.

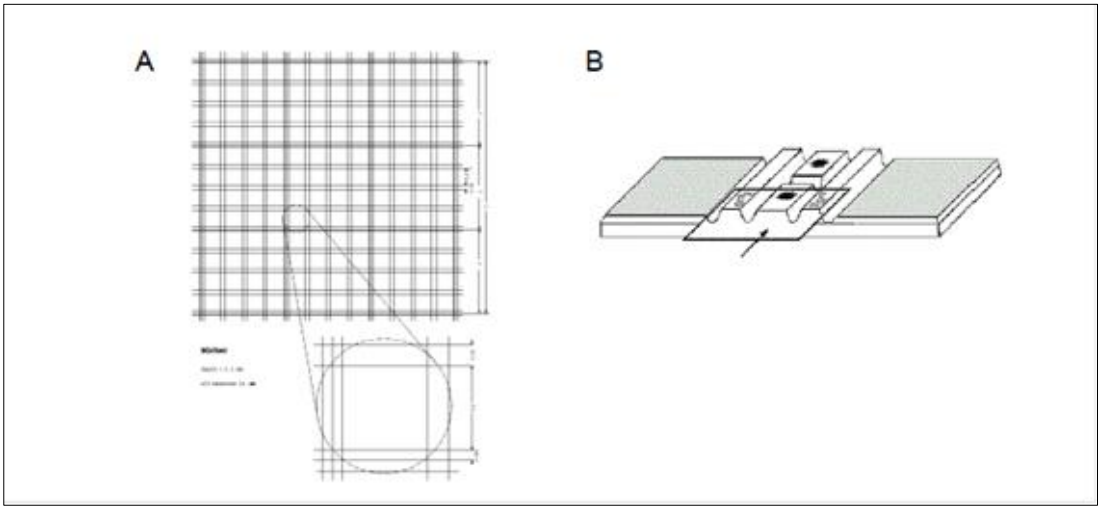


Figure 12. The Bürker chamber is a microscope slide with a grid pattern consisting of 9 fields, each of which is subdivided into 16 squares. (A). The counting area between the Bürker chamber and a coverslip placed on top of it is filled by capillarity with 10 µL of cell suspension and Trypan blue (B)

3.2 Genomic DNA Extraction

The starting material is a cell pellet of approximately $5*10^6$ lymphocytes from which to extract DNA. Blood contains several PCR amplification inhibitors, so a commercial kit (Cell lysis, protein precipitation 5PRIME) was used. The kit allows for cell membrane lysis, precipitation, and elimination of cellular proteins, followed by DNA precipitation using isopropanol and washing with ethanol. Finally, the DNA is resuspended in an aqueous solution, quantified using a spectrophotometer (SmartSpec 3000 Biorad), and stored in a freezer at -20°C. Diluted aliquots at 100 ng/µl are kept in the refrigerator for use over several months.

3.3 RNA Extraction

Cells were lysed by adding 1 ml of Trizol per $5x10^6$ lymphocytes and pipetting until complete lysis occurred. The Eppendorf was left at room temperature for 5 minutes. Then, 200 µl of Chloroform per 1 ml of Trizol used was added and manually and vigorously shaken for 15 seconds. The solution was allowed to sit at room temperature for another 5 minutes, followed by centrifugation at 12,000 rpm for 15 minutes at 4°C. At this point, the aqueous phase was collected and transferred to a new Eppendorf tube. Then, 500 µl of isopropanol per 1 ml of Trizol was added, mixed well to precipitate the RNA, and left at room temperature for 10 minutes or 1 hour/overnight at -20°C. Subsequently, centrifugation at 12,000 rpm for 15 minutes at 4°C was performed, and the Eppendorf was transferred to ice, removing the supernatant with a sterile syringe. 1 ml of cold 75% ethanol was added, followed by centrifugation at 12,000 rpm for 5 minutes at 4°C. The supernatant was removed, and the pellet was allowed to dry for 10 minutes at room temperature. Finally, the RNA pellet was resuspended in 5-20 µl of DEPC water (diethyl pyrocarbonate) depending on the starting cell quantity and the size of the RNA pellet obtained.

3.4 Reverse-Transcription PCR

To analyze only actively transcribed genes, RNA was reverse-transcribed into cDNA and then amplified by PCR for gene expression analysis. In the laboratory, the SuperScript II Reverse Transcriptase kit (Invitrogen) was used, which includes:

- SuperScript II reverse transcriptase (RNA-dependent DNA polymerase): it uses a single-stranded RNA strand as a template in the presence of primers to synthesize a complementary DNA strand (cDNA).

- RNase inhibitor: RNase is an enzyme responsible for degrading RNA present in the environment. Therefore, an RNase inhibitor is used to ensure the integrity of the extracted total RNA.

- Random hexamers: a mixture of 6-bp long primers that bind to randomly present complementary RNA sequences in the extracted RNA, forming a short double-stranded filament necessary for the initiation of reverse transcription.

- Deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) at 10mM.

- DTT 0.1M: a saline solution that enhances the efficiency of the enzyme.

- 5X PCR Buffer: a buffer containing KCl (500 mM) and TRIS-HCl (100 mM), necessary to maintain an appropriate pH for the reverse transcription reaction.

Initially, for each sample, 8µl of RNase-free water, 1µl of random hexamers (5µM), a total of 9µl, and 1µg of total RNA (2µl if concentrated at 0.5µg/µl) were added to the first reaction mix. The mix, kept on ice to preserve the RNA's integrity, was then incubated at 70°C for 3 minutes in a properly programmed thermocycler. To allow reverse transcription, another 9µl were added to the first mix, consisting of 4µl of 5X Buffer, 1µl of dNTPs (0.5 mM), 2µl of DTT (10 mM), 1µl of RNase inhibitor (40 U), and 0.5µl of reverse transcriptase (200 U). The total volume reached was 20µl. The sample was then subjected to the following temperatures in the thermocycler: 42°C for 60 minutes, 99°C for 5 minutes, and 4°C for 5 minutes.

3.5 Real-time PCR

Real-time PCR is a method used to quantify nucleic acids with high sensitivity and specificity. It's called "real-time" because it allows researchers to observe the increase in DNA quantity during its amplification. This is possible because it detects and quantifies fluorescent molecules: these compounds bind to the amplified DNA and emit a signal that increases proportionally with the increase in amplified products. You obtain an amplification curve where the number of cycles is on the x-axis, and fluorescence normalized to an internal fluorophore is on the y-axis.

At the beginning of the reaction, there are only small changes in fluorescence, and this is called the "baseline region." The increase in fluorescence above this threshold indicates the formation of amplification products. From this point on, the reaction will enter an exponential phase that degenerates into a plateau at the end of the reaction [25, 26]. In the middle of the cycles, the curve has a linear run: this represents the most important phase in this stage where the amount of amplified cDNA correlates with the amount of cDNA expressed at the beginning in the sample. In this linear region, a fluorescence threshold is chosen: from this value, it is possible to obtain the Ct (cycles threshold), which is the number of cycles needed for that sample to reach that emission threshold (Figure 13).

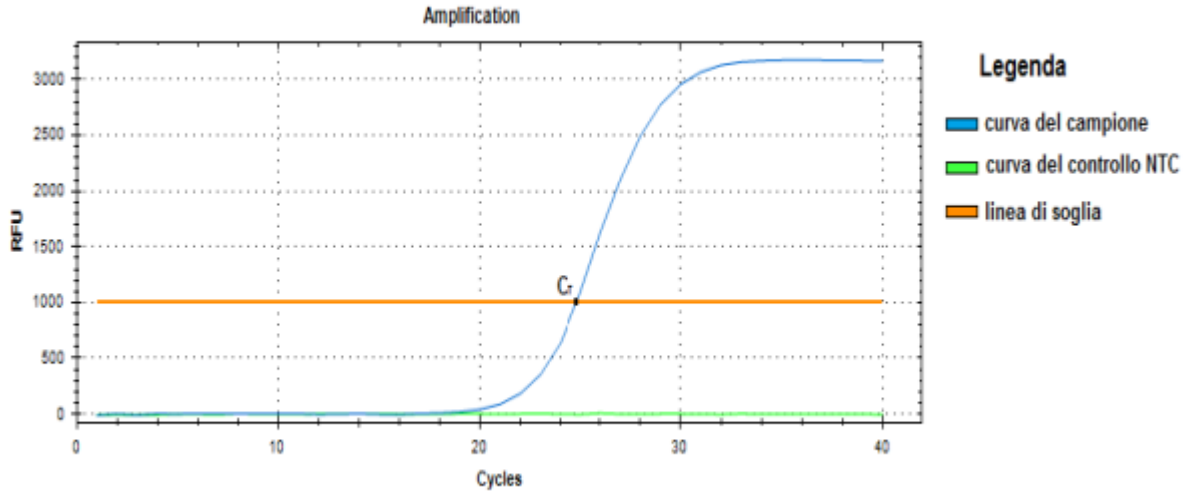


Figure 13 - Example of an amplification curve for a sample and a No-Template Control (NTC)

We used SYBR GREEN as the detector. Its molecules emit low levels of fluorescence when present in the solution; however, the signal becomes stronger when the fluorophore binds to double-stranded DNA. Nevertheless, SYBR GREEN is not a selective fluorophore and binds to all DNA, as well as primer dimers. This is why it's recommended to introduce an additional step after amplification, known as the dissociation protocol: the temperature gradually increases until all double-stranded DNA is denatured.

This method allows the identification of contaminants or nonspecific amplification products since they exhibit different melting temperatures. There's also a second fluorophore called ROX, which functions as an internal reference used by the instrument to normalize SYBR GREEN fluorescence.

For evaluating the expression of the target gene, we will choose a relative quantification method using the ΔΔCt formula:

1) ΔCt = Ct (target gene) - Ct (reference gene)

2) ΔΔCt = ΔCt (of the patient of interest) - the median of the ΔCt values of the controls analyzed.

3) 2^-ΔΔC

The "2" value in the last formula represents the highest efficiency for the reaction, indicating that the product doubles with each amplification cycle.

We used Sequence Detection System 7000 (Applied Biosystem) thermal cycler and the ABI PRISM 7000 software. The PCR mix used is as follows:

- Platinum SYBR GREEN master mix: 7.5µl
- Forward primer (10pmol/µl): 1µl
- Reverse primer (10pmol/µl): 1µl
- H2O: 4.5µl
- cDNA: 1µl
- Final volume: 15µl

The amplification protocol consists of the following stages:

Stage 1: 50°C for 2 minutes

Stage 2: 95°C for 10 minutes

Stage 3: 95°C for 15 seconds

Stage 4: 60°C for 1 minute

Stage 5: 95°C for 15 minutes

This is followed by a dissociation stage to eliminate any potential contaminants or primer dimers: - 60°C for 20 seconds, followed by 95°C for 15 seconds.

The primer sequences used in real-time PCR were identified using the Primer Express program (Applied Biosystem).

Table2. *Primers for Real-time PCR:*

BRCA1	**FORWARD**	**REVERSE**
Esoni 14-15	ttgttgatgtggaggagcaa	gattccaggtaaggggttcc
BRCA2	**FORWARD**	**REVERSE**
Esoni 15-16	aacaaaggcaacgcgtctt	cgtttttgtaggtgagacgg

3.6 Qualitative PCR and Sanger Sequencing

The sequencing of NBS1 was performed using the Polymerase Chain Reaction (PCR) with specific primers and suitable melting temperatures (see Table 3). The products obtained from this amplification were directly sequenced using the Abi Prism-310 Genetic Analyzer from Applied Biosystem. The analysis of the obtained sequences was conducted using specific software, BLAST (www.blast.ncbi.nlm.nih.gov), and mutations were confirmed using the ENSEMBL genome browser (www.ensembl.org).

NBS1	**FOWARD**	**REVERSE**	**Tm Annealing**
Esoni 3-4-5-6	gatgaaaatccctgtattgacattaaaaga	ctctgtttggcattcaaaaatataaatgt	54

Table 3. *Primers for exons 3-4-5-6 NBS1 gene*

This PCR protocol you've provided is as follows:

- Initial denaturation: 94°C for 2 minutes
- Denaturation: 94°C for 30 seconds
- Annealing: 54°C for 30 seconds
- Extension: 72°C for 30 seconds
- Final extension: 72°C for 7 minutes

After this PCR amplification, the product will be analyzed using agarose gel electrophoresis (2%), as shown in Figure 11, to confirm the success of the amplification reaction. Subsequently, the PCR product will be subjected to Sanger sequencing.

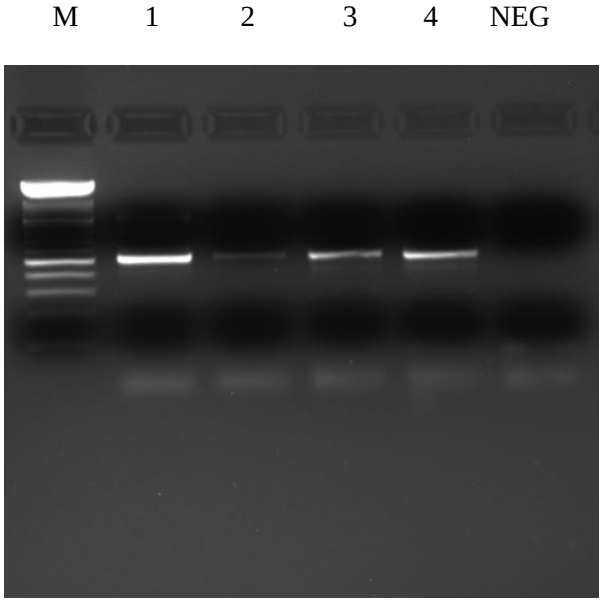


Figure 14. A picture shows of the gel after electrophoresis of the amplification products for the identification of the NBS1 gene. In this gel, it appears that there are four positive cases (lanes 1, 2, 3, 4) which exhibit a band of 531 base pairs (bp). The "M" likely stands for marker, and "NEG" typically indicates the negative control.

After PCR amplification, the product is purified, and then Big Dye Terminator is used to label the nucleotides. This sample is further purified to remove excess Big Dye, and finally, the purified sample is loaded into the ABI PRISM 3130 Genetic Analyzer sequencer to generate the nucleotide sequence.

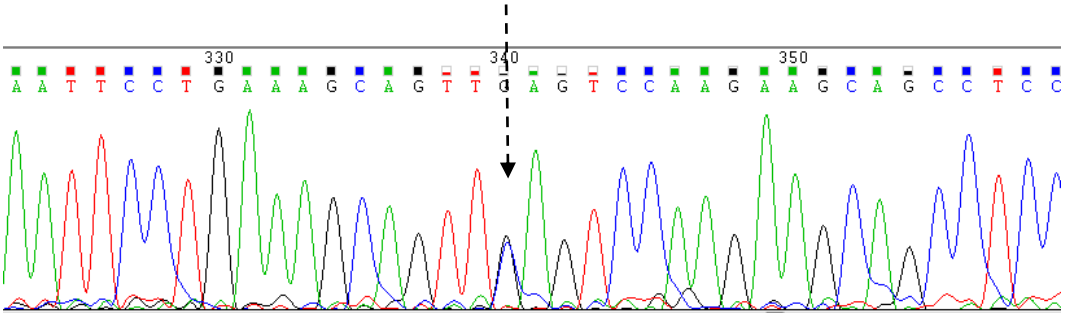


Figure 14 The nucleotide sequence of NBS1 generated through Sanger sequencing showing positivity for SNP rs1805794 is a specific genetic sequence.

3.7 Multiplex ligation-dependent probe amplification (MLPA)

The Multiplex ligation-dependent probe amplification (MLPA) is a rapid and sensitive technique used to quantify the copy number of over 50 DNA or RNA sequences in a single PCR reaction using a single pair of primers. This method uses two oligonucleotide probes for each target of interest. These probes are designed to be contiguous to the nucleotide region to be analyzed and contain 3' and 5' sequences that serve as primers (sequence X and sequence Y), which are the same for all the probes being examined. One of the two primers is conjugated with a fluorescent marker. Furthermore, one of the probes contains a variable-length sequence (stuffer) for the simultaneous analysis of multiple targets, allowing discrimination of sequences that may differ by just a single nucleotide. The uniqueness of MLPA is that it is the probes added to the genomic samples that are amplified and quantified. The technique involves five distinct steps: 1) denaturation of DNA and hybridization with oligonucleotide probes; 2) ligation reaction; 3) PCR reaction; 4) separation of PCR products by electrophoresis; 5) analysis of the results (Figure 15).

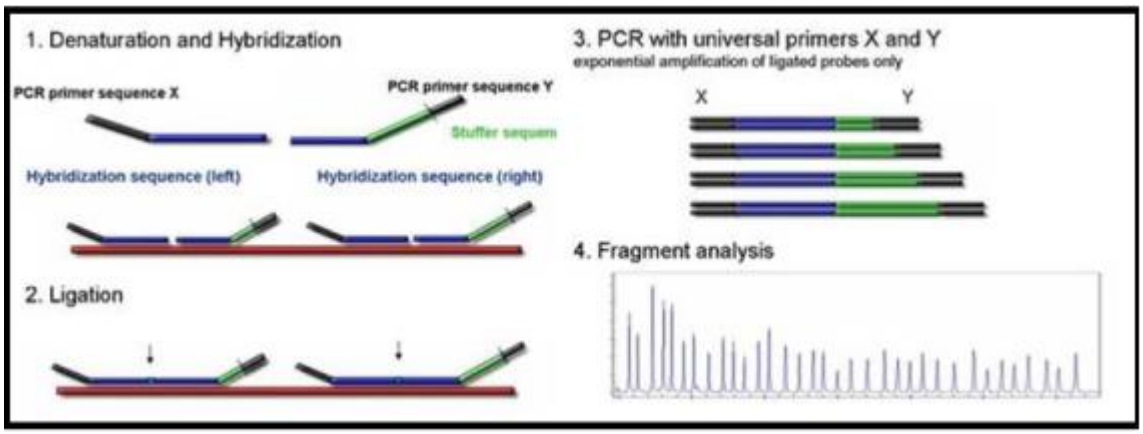


Figure 15. The Multiplex ligation-dependent probe amplification (MLPA) method

MLPA Protocol*

1. *Sample Preparation:*

- DNA samples should have a concentration of approximately 100 ng/µl.

- In PCR tubes, add x µl of DNA and 5-x µl of TE for a final volume of 5 µl, where x = (100 ng/µl)/[DNA].

2. *Denaturation:*

- Place the tubes in a thermocycler and run the following program:

  - 98°C for 10 minutes

  - 25°C for 10 minutes

  - 95°C for 1 minute

  - 60°C for 16 hours

- 54°C (infinite)

- At 25°C, add 1.5 µl of MLPA Buffer and 1.5 µl of SALSA Probe-mix to each sample. Incubate for 16 hours.

3. *Ligation:*

- Prepare the ligation mix for each sample using 3 µl of Ligase-65 buffer A, 3 µl of Ligase buffer B, 1 µl of Ligase-65, and 25 µl of distilled water.

- Block the previous program and start a new one without removing the tubes from the thermocycler:

  - 54°C for 15 minutes

  - 98°C for 5 minutes

  - 4°C (infinite)

- At 54°C, add 32 µl of the ligation mix to each sample.

4. *PCR Reaction:*

- Dispense 10 µl of the ligation product into new PCR tubes and add 4 µl of SALSA PCR-buffer

10X and 26 µl of distilled water for each sample.

- Start the PCR reaction under the following conditions:
  - 60°C for 15 minutes
  - 95°C for 30 seconds
  - 60°C for 30 seconds (33 cycles)
  - 72°C for 1 minute
  - 72°C for 20 minutes
  - 4°C (infinite)

-Prepare the PCR mix using 2µl of SALSA PCR primer, 2µl of SALSA Enzyme dilution buffer,0.5µl of SALSA polymerase, and 5.5µl of distilled water for each sample.

- At 60°C, add 10µl of the mix to each sample.
- After the PCR reaction, add 0.5µl of ROX, 12µl of formamide, and 0.75µl of distilled water to 1µl of the amplification product.
- Denature the samples at 94°C for 3 minutes, 20°C for 7 minutes, and cool to 4°C.
- Separate the PCR products using the ABI Prism 310 automatic sequencer.
- Analyze the results using the Coffalyser software.

In each experiment, subjects to be tested are compared with control subjects who do not have deletions or duplications within the gene under study. The first phase involves normalizing the signal within each sample. The area of peak X is divided by the sum of the areas of all peaks in that mix, and this is done for both test and control samples. Normalized signals can be compared with each other by calculating the ratio of the area of peak X in the sample to the average value of peak X in the controls. A ratio close to 1 indicates no variation in the copy number of the target sequence between the sample and control. However, if the ratio significantly deviates from 1 (≤ 0.5 or ≥ 1.5), it indicates a variation in the copy number of the target sequence compared to the control.

3.8 Cell lines

Cell lines are monoclonal populations of immortalized cells that are able to continually proliferate in culture without undergoing senescence. These continuous cell lines are particularly useful in research settings as they provide a virtually unlimited supply of homogeneous cell material for genetic and biochemical studies. Human cell lines are difficult to develop even from cancerous tissues despite this apparent hindrance more than 1000 human hematopoietic cell lines have been generated since the 1960s by setting up long term cell culture from bone marrow (BM), peripheral blood (PB) or pleural effusions of ALL, AML or lymphoma patients. Whereas normal hematopoietic cells can only survive in vitro for days to weeks, these cell lines are able to proliferate continuously in culture while preserving the majority of their characteristic genetic alterations and phenotypic features. Cell lines carrying many of the key translocations and oncogenes implicated in human leukemogenesis are established over the past years. When leukemic cell lines bearing an oncogene of interest are not readily available, an alternate approach can be followed to introduce this gene into a cell line and to characterize the effects on proliferation, differentiation and intracellular signaling. Cell lines derived from sources other than human hematopoietic malignancies have also been used to study the functional consequences of oncogene expression. For example, the human B-precursor ALL cell lines, MHH-CALL-4 and REH were purchased from the DSMZ. MHH-CALL-4 has an *IGH@-CRLF2* translocation and a JAK2 I682F mutation. and REH are $CRLF2_{wt}$ B-precursor ALL cell lines with a t(12;21), that were used as controls. Cell lines were maintained in RPMI with FBS and penicillin/streptomycin/glutamine in a 37°C incubator with 5% CO_2 and harvested in the logarithmic phase of growth with > 95% viability.

Chapter 4. Aberrations of NBS1, Fancd2, Palb2 Genes and Expression of BRCA1 and BRCA2

4.1 Specific background and aims.

Our research endeavors adhered to the highest ethical standards, having been approved by the Local Clinical Research Ethics Committee in Catania, Italy. Every step of our study was conducted with the utmost respect for ethical considerations, and written informed consents were diligently obtained from the parents of each child participating in this research.

Our study scrutinized bone marrow (BM) samples obtained during the diagnosis and remission (Dx/Rem) phases of 111 children who had been diagnosed with B-cell acute lymphoblastic leukemia (B-ALL). These patients were cared for at the Center of Pediatric Hematology Oncology in Catania, Italy, over the span of 17 years, from 2000 to 2017. They were enrolled in three consecutive protocols of the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP), namely AIEOP-BFM ALL 2000, AIEOP-R 2006, and AIEOP BFM ALL 2009.

Our research cohort represented a diverse spectrum of biological subgroups. This diversity enabled us to investigate the impact of genetic factors on treatment response across various B-ALL subtypes. The subgroups included children presenting 16 with t(1;19), 52 with t(12;21), 9 with t(9;22)/Ph+, 5 with a KMT2A (formerly MLL) rearrangement, and 32 categorized as "B-Others." It was this heterogeneity that allowed us to glean a comprehensive understanding of the genetic landscape of B-ALL.

Our meticulous investigation of genetic mutations involved the application of multiple sophisticated techniques. To determine NBS1 mutations in exons 3-6, we employed traditional PCR methodology, which was followed by Sanger sequencing for precise characterization. The genetic alterations associated with Fanconi anemia complementation group D2 (FANCD2) and partner and localizer of BRCA2 (PALB2) were explored through multiplex ligation-dependent probe amplification (MLPA). To establish a baseline for these mutations, samples from healthy donors were used as wild-type controls.

Our study ventured beyond genetic mutations and delved into the realm of gene expression. Using a real-time PCR assay with SYBR Green, a fluorescent dye, we meticulously evaluated the expression of the BRCA1 and BRCA2 genes. This approach allowed us to quantify the gene expression levels for specific segments, namely BRCA1 exons 14-15 and BRCA2 exons 15-16. The gene expression data was stratified into two categories: high (H) and low (L), a differentiation that was made based on the Mean and Median of fold change (FC) as the critical thresholds.

In conclusion, our research represents a comprehensive endeavor that not only delves into the genetic underpinnings of B-ALL but also adheres to the highest ethical standards. The study inclusion of diverse biological subgroups and the meticulous application of advanced genetic techniques has enriched our understanding of the complex interplay between genetics and treatment outcomes in pediatric leukemia. This valuable knowledge paves the way for the development of more personalized and effective treatment strategies, offering hope to young patients facing the challenges of B-ALL. However, as we conclude this phase of the research, it becomes evident that there is much more to explore and uncover. As we look to the future, prospective projects with larger populations beckon, offering the promise of deeper insights into the genetic intricacies of this disease.

Most notably, the study identified a statistically significant association between the expression levels of both BRCA1-H and BRCA2-H and relapse. Three children with BRCA1-H/BRCA2-H had experienced a relapse out of six cases, in contrast to eight out of 65 cases with BRCA1-L/BRCA2-L. These findings suggest that genetic variations in NBS1 and the expression levels of BRCA genes may have a significant impact on the susceptibility to severe toxicity and relapse in pediatric B-ALL patients undergoing chemotherapy. Further research is warranted to delve deeper into these genetic associations, which may hold the key to more personalized and effective treatment strategies for B-ALL.

Table 3. ***NBS1, FANCD2, PALB2*** **alterations and** ***BRCA1, BRCA2*** **expression at diagnosis and remission in patients with t(1;19) positive B-ALL**.

Cases	***NBS1***		***FANCD2***		***PALB2***		***BRCA1***		***BRCA2***	
t(1;19)ALL	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**
CT1	rs758830069 Δ119	rs758830069 Δ119	wt	N.A.	wt	N.A	L	L	L	L
CT2	wt	N.A.	Δ35	wt	Ex. 1 Dupl.	wt	H	L	H	H
CT3	wt	N.A.	wt	N.A.	wt	N.A	H	L	H	H
CT4	wt	N.A.	Ex.1 Dupl.	wt	Ex. 12 Dupl.	wt	H	L	L	H
CT5	rs1805794 G>C	rs1805794 G>C	wt	N.A.	wt	N.A	H	H	H	L
CT6	wt	N.A.	Ex.1 Dupl.	wt	Ex.7_9_10_12Dupl.	wt	H	H	H	H
CT7	rs1805794 G>C	rs1805794 G>C	wt	N.A.	wt	N.A	L	H	L	L
CT8	rs1805794 G>C	rs1805794 G>C	Ex.1 Dupl.	wt	wt	N.A	H	H	L	L
CT9	wt	N.A.	wt	N.A.	Δ 1	wt	L	H	H	H
CT10	rs1805794 G>C	rs1805794 G>C	wt	N.A.	wt	N.A	H	L	L	L
CT11	wt	N.A.	wt	N.A.	wt	N.A.	H	H	L	L
CT12	wt	N.A.	wt	N.A.	wt	N.A.	L	L	L	L
CT13	wt	N.A.	wt	N.A.	wt	N.A.	H	L	L	L
CT14	wt	N.A.	wt	N.A.	wt	N.A.	H	L	H	L
CT15	wt	N.A.	wt	N.A.	wt	N.A.	L	L	H	L
CT16	wt	N.A.	wt	N.A.	wt	N.A.	L	L	H	L

Analysis of *NBS1* was detected at diagnosis a missense variant mutation (G>C;Glu185Gln) in 5 patients (31.3%); in one case had exon-4 deletion. *FANCD2* analysis showed mutations in 4 patients (26%) at diagnosis and wild-type mutation at remission; *PALB2* genomic aberrations was detected in 4 cases (25%) at diagnosis and wild-type mutation at remission. Additional *BRCA1* and *BRCA2* expression analyzed in 16 patients at diagnosis/remission. *BRCA1-high* were 10 out of 16 with mean FC 17,239 (62,5%) and *BRCA2-high* were 8 out of 16 with mean FC 2,911 (50%). In remission, *BRCA1-high* were 6 out of 16 with mean FC 7.625 (37.5%) and *BRCA2-high* were 5 out of 16 with mean FC 5,482 (31,3%).

Abbreviations: wt indicates wild-type; Δ, deletion; Dupl., duplication; Ex., exon; N.A.; not analyzed because patients with wild-type gene mutation at diagnosis; H., high expression , L., low expression

Table 4. ***NBS1, FANCD2, PALB2*** **alterations and** ***BRCA1, BRCA2*** **expression at diagnosis and remission in patients with t(12;21) positive B-ALL**.

Cases	***NBS1***		***FANCD2***		***PALB2***		***BRCA1***		***BRCA2***	
t(12;21)ALL	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**
CT12	wt	N.A.	wt	N.A.	wt	N.A.	L	H	L	H
CT18	rs1805794 G>C	rs1805794 G>C	wt	N.A.	wt	N.A.	L	L	H	L
CT19	wt	N.A.	wt	N.A.	wt	N.A.	H	L	H	L
CT20	wt	N.A.	wt	N.A.	wt	N.A.	H	L	L	L
CT21	rs1805794 G>C	rs1805794 G>C	Δ29/35	wt	Δ2-3	wt	H	L	L	L
CT22	rs1805794 G>C	rs1805794 G>C	wt	N.A.	wt	N.A.	L	L	L	L
CT23	rs1805794 G>C	rs1805794 G>C	wt	N.A.	wt	N.A.	L	L	L	L
CT24	wt	N.A.	Δ1	wt	Δ10	wt	H	H	L	L
CT245	rs1805794 G>C	rs1805794 G>C	wt	N.A.	wt	N.A.	H	L	L	L
CT26	rs1805794 G>C	rs1805794 G>C	wt	N.A.	wt	N.A.	L	L	L	L
CT27	wt	N.A.	wt	N.A.	wt	N.A.	L	H	L	H
CT28	wt	N.A.	wt	N.A.	wt	N.A.	L	L	H	L
CT29	wt	N.A.	wt	N.A.	wt	N.A.	H	L	L	L
CT30	wt	N.A.	wt	N.A.	wt	N.A.	L	L	L	L
CT31	wt	N.A.	wt	N.A.	wt	N.A.	H	H	L	L
CT32	wt	N.A.	wt	N.A	wt	N.A	L	L	H	L
CT33	wt	N.A.	Δ1	wt	Δ10	wt	L	L	L	L
CT34	wt	N.A.	wt	N.A	wt	N.A	H	H	L	L
CT35	wt	N.A.	wt	N.A	wt	N.A	L	L	H	H
CT36	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	H	L	L	L
CT37	wt	N.A.	wt	N.A	wt	N.A	H	L	H	L
CT38	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	L	L	L	L
CT39	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	L	L	L	L

CT40	rs1805794 G>C	rs1805794 G>C	Δ1	wt	Δ6-10	wt	L	L	L	L
CT41	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	L	L	L	L
CT42	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT43	wt	N.A.	wt	N.A	wt	N.A	H	H	L	L
CT44	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT45	wt	N.A.	wt	N.A	wt	N.A	L	L	H	H
CT46	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	L	L	L	L
CT47	wt	N.A.	wt	N.A	wt	N.A	H	L	L	L
CT48	wt	N.A.	wt	N.A	wt	N.A	H	L	L	L
CT49	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT50	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	H	H	H	H
CT51	wt	N.A.	Δ29	wt	Δ35	wt	L	L	L	L
Cases	***NBS1***		***FANCD2***		***PALB2***		***BRCA1***		***BRCA2***	
t(12;21)ALL	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**
CT52	wt	N.A.	wt	N.A	wt	N.A	L	L	H	H
CT53	wt	N.A.	wt	N.A	wt	N.A	H	H	L	L
CT54	wt	N.A.	wt	N.A	wt	N.A	H	L	L	L
CT55	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	L	L	L	L
CT56	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	H	L	L	L
CT57	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT58	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT59	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	L	L	L	L
CT60	wt	N.A.	wt	N.A	wt	N.A	L	L	H	L
CT61	wt	N.A.	wt	N.A	wt	N.A	H	L	L	L
CT62	wt	N.A.	wt	N.A	wt	N.A	L	L	H	L
CT63	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT64	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT65	wt	N.A.	wt	N.A	wt	N.A	H	L	H	L

Analysis of *NBS1* was detected at diagnosis a missense variant mutation (G>C;Glu185Gln) in 16 (30%) out of 53 patients at diagnosis and remission. Deletions for *FANCD2* and *PALB2* genes were found at diagnosis in 5 patients. Additional *BRCA1* and *BRCA2* expression in 53 patients at diagnosis and remission. 18 patients (34%) presented an expression higher *BRCA1* than HDs with mean FC 13,63 and 11 patients (24%) presented an expression higher *BRCA2* with mean 1,59. In remission, 8 patients (15%) an expression higher *BRCA1* and 6 patients an expression higher *BRCA2* than HDs with mean FC 5,631 and mean FC 3,23, respectively.

Abbreviations: wt indicates wild-type; Δ, deletion; Dupl., duplication; Ex., exon; N.A.; not analyzed because patients with wild-type gene mutation at diagnosis. N.E, not evaluable; H., high expression, L., low expression.

Table 5. ***NBS1, FANCD2, PALB2*** **alterations and** ***BRCA1, BRCA2*** **expression at diagnosis and remission in patients with t(9;22) positive B-ALL**.

Cases	***NBS1***		***FANCD2***		***PALB2***		***BRCA1***		***BRCA2***	
t(9;22)ALL	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**
CT66 Rel	wt	N.A.	wt	N.A.	wt	N.A.	L	H	L	H
CT67	wt	N.A.	Δ35-32	Δ2	Δ2-6	wt	L	H	L	H
CT68	wt	N.A.	wt	N.A.	wt	N.A.	L	L	L	L
CT69	rs1805794 G>C	rs1805794 G>C	wt	N.A.	wt	N.A.	H	L	H	H
CT70	wt	N.A.	Δ10	wt	wt	N.A.	H	H	L	H
CT71	wt	N.A.	Δ38	wt	Δ2	wt	H	L	H	L
CT72	wt	N.A.	wt	N.A.	wt	N.A.	L	H	H	L
CT73	wt	N.A.	wt	N.A.	wt	N.A.	H	L	H	L
CT74	rs1805794 G>C	rs1805794 G>C	wt	N.A.	wt	N.A.	H	L	H	L

Analysis of *NBS1* was detected at diagnosis a missense variant mutation (G>C;Glu185Gln) in 2 patients (22,2%). *FANCD2* analysis showed deletions in 3 patients (33,3%); *PALB2* genomic aberrations at diagnosis was detected in 2 cases (22,2%). Additional *BRCA1* and *BRCA2* expression in 5 patients with t(9;22) B-ALL at diagnosis, one patient at relapse and remission. *BRCA1-high* were 5 out of 9 with mean *BRCA1* 8,915 (55,5%) and *BRCA2-high* were 5 out of 9 with mean *BRCA2* 2,413 (55,5%). In remission, four patients (44,4%) presented an expression higher *BRCA1* and *BRCA2* than HDs with mean FC 6,229 and 4,583, respectively.

Abbreviations: wt indicates wild-type; Δ, deletion; Ex., exon; N.A.; not analyzed because patients with wild-type gene mutation at diagnosis; rel., relapse; H., high expression, L., low expression.

Table 6. ***NBS1, FANCD2, PALB2*** **alterations and** ***BRCA1, BRCA2*** **expression at diagnosis and remission in others B-ALL subgroup.**

Cases	***NBS1***		***FANCD2***		***PALB2***		***BRCA1***		***BRCA2***	
Others-B	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**
CT75	wt	N.A.	wt	N.A	Ex. 2 Dupl.		L	H	H	L
CT76	wt	N.A.	wt	N.A	wt	N.A	L	L	L	H
CT77	wt	N.A.	wt	N.A	wt	N.A	L	L	H	H
CT78	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	H	L	L	L
CT79	wt	N.A.	wt	N.A	wt	N.A	H	L	H	H
CT80	wt	N.A.	Ex. 2-29 Dupl.; Δ9-30	wt	Δ 6-8	wt	H	N.A.	H	N.A.
CT81	wt	N.A.	wt	N.A	wt	N.A	H	L	L	L
CT82	wt	N.A.	Ex.1-2_Dupl.	wt	Ex.12 Dupl; Δ6	wt	H	H	H	L
CT83	rs1805794 G>C	rs1805794 G>C	Ex. 1 Dupl.	wt	Ex.12 Dupl.	wt	H	H	L	L
CT84	rs1805794 G>C	rs1805794 G>C	Ex. 1 Dupl.	wt	wt	N.A	H	H	L	L
CT85	wt	N.A.	wt	N.A	wt	N.A	H	H	H	H
CT86	wt	N.A.	Ex. 10 Dupl.	wt	wt	N.A	L	L	L	H
CT87	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	L	L	L	L
CT88	wt	N.A.	Ex.10 Dupl.	wt	wt	N.A	H	H	L	L
CT89	wt	N.A.	wt	N.A	wt	N.A	L	L	H	H
CT90	wt	N.A.	wt	N.A	wt	N.A	H	L	H	L
CT91	wt	N.A.	wt	N.A	wt	N.A	H	L	L	L
CT92	wt	N.A.	wt	N.A	wt	N.A	H	L	L	L
CT93	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT94	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L

CT95	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT96	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	L	L	H	L
CT97	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT98	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT99	wt	N.A.	wt	N.A	wt	N.A	L	L	H	L
CT100	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	L	L	L	L
CT101	rs1805794 G>C	rs1805794 G>C	wt	N.A	wt	N.A	L	L	L	L
CT102	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT103	wt	N.A.	wt	N.A	wt	N.A	L	L	H	L
CT104	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT105	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L
CT106	wt	N.A.	wt	N.A	wt	N.A	H	L	H	L
CT107	wt	N.A.	wt	N.A	wt	N.A	L	L	L	L

Analysis of *NBS1* was detected at diagnosis a missense variant mutation (G>C;Glu185Gln) in 7 patients (22%). *NBS1* mutations were detected in remission samples showing a germline genomic aberration. *FANCD2* analysis showed duplication (exons-1-2-10-29) in 6 patients (19%) and in one patient also observed a deletion in 9-30 exons; *PALB2* genomic aberrations was detected in 3 cases (10%). Additional *BRCA1* and *BRCA2* expression in 32 patients. *BRCA1-high* were 13 out of 32 with mean *BRCA1* 13,278 (41%) and *BRCA2-high* were 12 out of 32 with mean *BRCA2* 1,5 (37,5%).%). In remission, 6 patients (19%) presented an expression higher *BRCA1* than HDs with mean FC 2.130 and 6 patients (19%) presented an expression higher *BRCA2* with mean 3,277.

Abbreviations: wt indicates wild-type; Δ, deletion; Dupl., duplication; Ex., exon; N.A.; not analyzed because patients with wild-type gene mutation at diagnosis; H., high expression, L., low expression.

Table 7. ***NBS1, FANCD2, PALB2*** **alterations and** ***BRCA1, BRCA2*** **expression at diagnosis and remission in MLL rearranged B-ALL subgroup**.

Cases	***NBS1***		***FANCD2***		***PALB2***		***BRCA1***		***BRCA2***	
Dx	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**	**Dx**	**Rem**
CT108	wt	N.A.	wt	N.A.	wt	N.A.	H	L	H	H
CT109	rs1805794 G>C	rs1805794 G>C	wt	N.A.	wt	N.A.	L	H	L	H
CT110	wt	N.A.	wt	N.A.	Ex. 1_Dupl.	wt	L	L	L	L
CT111	rs1805794 G>C	rs1805794 G>C	wt	N.A.	wt	N.A.	H	H	H	L
CT112	wt	N.A	wt	N.A	wt	N.A	L	H	L	H

Analysis of *NBS1* was detected at diagnosis and remission a missense variant mutation (G>C;Glu185Gln) in 2 patients (40%). Alterations in *FANCD2* was not found; *PALB2* genomic aberrations was detected in 1 case (20%) at diagnosis. *BRCA1-high* were 2 out of 5 with mean *BRCA1* 40,254 (40%) and *BRCA2-high* were 2 out of 5 with mean *BRCA2* 0,432 (40%). In remission, two patients presented an expression higher *BRCA1* than HDs with mean FC 5,278 and three patients presented an expression higher *BRCA2* with mean FC 28,787.

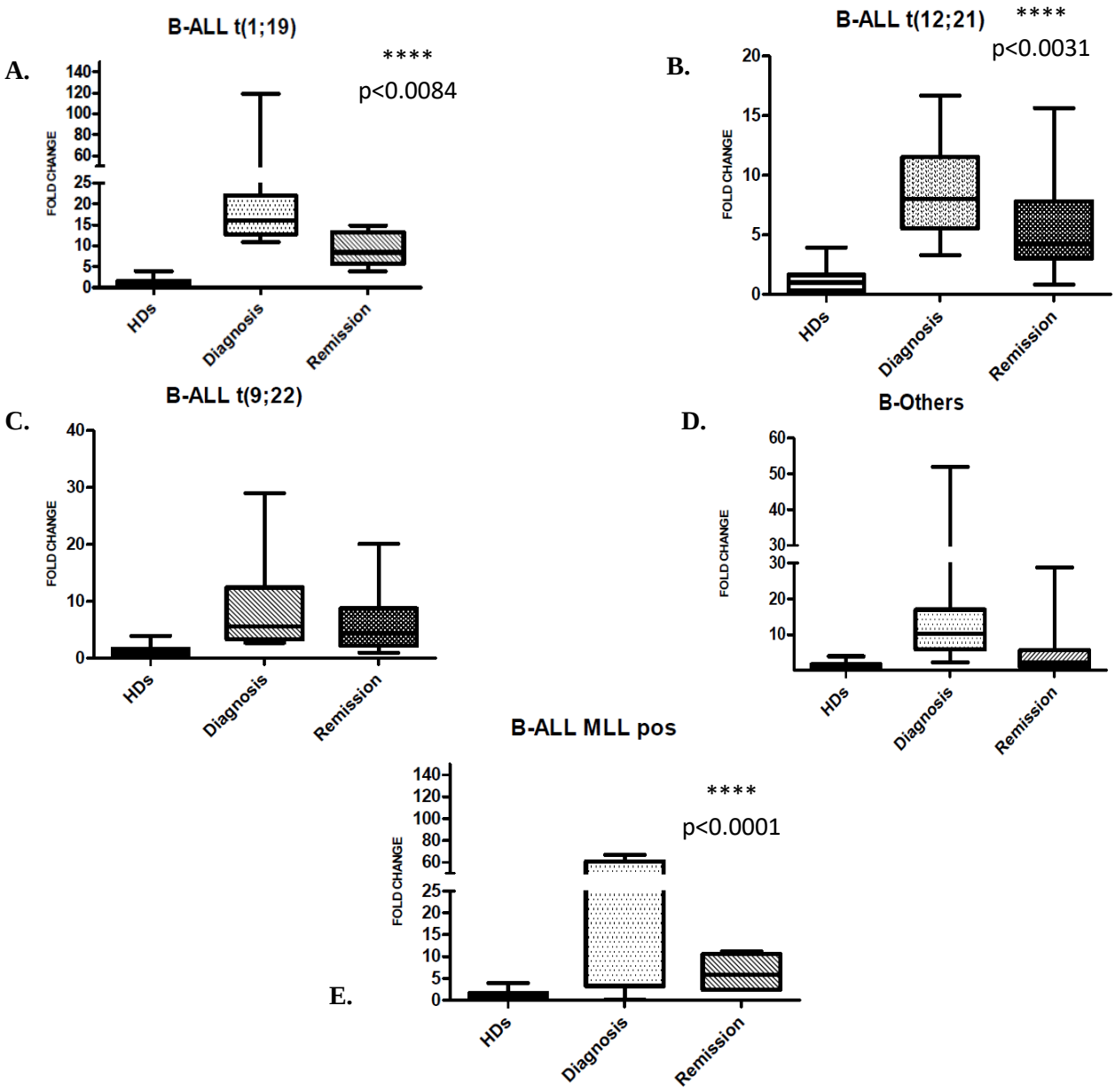
Abbreviations: wt indicates wild-type; ex., exon; dupl., duplication; N.A.; not analyzed because patients with wild-type gene mutation at diagnosis; H., high expression, L., low expression.

Table 8. NBS1, FANCD2, PALB2 alterations and BRCA1, BRCA2 expression in B-ALL cell lines CALL4 and REH.

Cell lines	*NBS1*	*FANCD2*	*PALB2*	*BRCA1*	*BRCA2*
CALL4	wt	wt	wt	H	H
REH	rs1805794 G>C	wt	wt	H	H

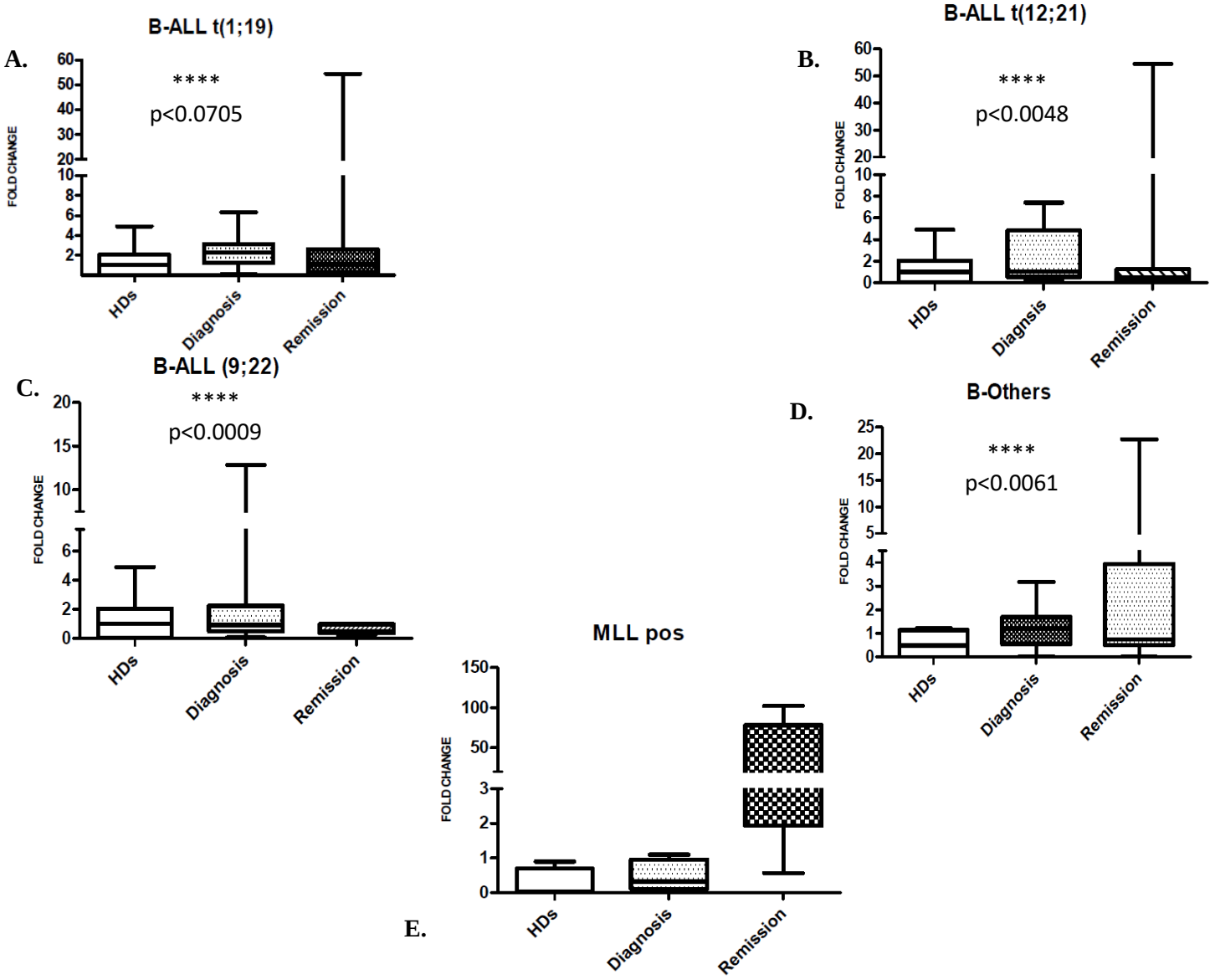
4.2 Comparison of *BRCA*1 expression at diagnosis and remission in ALL subgroups vs Healthy Donors (HD)

For each specimen, results are reported as fold changes on the median expression value of their respective cohort. **A.** B-ALL patients with t(1;19) at diagnosis vs HDs ($p<0.0293$) and comparison BRCA1 expression in patients at remission; **B.** B-ALL patients with t(12;21) vs HDs ($p<0.0001$) and in patients at remission; **C**. B-ALL with t(9;22) at diagnosis ($p<0.0084$) and remission vs HDs; **D**. Others B-ALL at diagnosis ($p<0.0031$) and remission vs HDs; **E.** B-ALL with MLL rearranged at diagnosis and remission ($p<0.0001$) vs HDs; (HD=healthy donor).



4.3 Comparison of *BRCA*2 expression at diagnosis and remission in ALL subgroups vs Healthy Donors (HD)

For each specimen, results are reported as fold changes on the median expression value of their respective cohort. A. B-ALL with t(1;19) at diagnosis ($p<0.0705$) and remission vs HDs; B. B-ALL with t (12;21) at diagnosis ($p<0.0048$) and remission vs HDs; C. B-ALL patients with t(9;22) at diagnosis and remission vs HDs; D. Others B-ALL at diagnosis ($p<0.0061$) and remission vs HDs; (HD=healthy donor); E. B-ALL patients at diagnosis and remission with MLL rearranged vs HDs



4.4 Summary and conclusions

In this study, involving a cohort of 111 patients, several significant findings shed light on the relationships between genetic polymorphisms and outcomes in pediatric B-cell acute lymphoblastic leukemia (B-ALL) patients undergoing chemotherapy.

First, it was observed that almost half of the patients (48%) experienced more than one episode of severe toxicity during their chemotherapy treatment. Additionally, 19% of the children with B-ALL in this study had experienced disease recurrence.

The study focused on the NBS1 polymorphism, specifically the rs1805794 SNP_G>C, which was found in 28% of the patients. Strikingly, this genetic variation appeared to play a protective role against severe toxicity. The data indicated that only 10 out of the 54 patients who had suffered from high-grade toxicity had this particular polymorphism. Importantly, this protective effect was more pronounced in the subgroup of patients with t(12;21)-positive B-ALL.

However, the study did not establish any statistically significant associations between the NBS1 polymorphism, relapse rates, or the presence of FANCD2/PALB2 aberrations and B-ALL toxicity or recurrence.

At the time of diagnosis, the study revealed another intriguing correlation. Children who exhibited high expression of both BRCA genes, that is BRCA1 and BRCA2 (BRCA1-H/BRCA2-H), were found to be at an increased risk of experiencing severe toxicity. In fact, 17 out of 27 patients with BRCA1-H/BRCA2-H faced severe toxicity compared to only 10 out of 28 patients with lower BRCA gene expression levels (BRCA1-L/BRCA2-L).

Furthermore, during remission, it was noted that high BRCA1 expression (BRCA1-H) was associated with a higher risk of relapse, though this relationship did not reach statistical significance. On the other hand, the higher expression of BRCA2 (BRCA2-H) was linked to a significantly increased risk of recurrence compared to patients with lower BRCA2 expression (BRCA2-L).

Whole ALL (n° 111)	n°	NBS1 poly (+)	NBS1 poly (-)	*p-value*
		32	79	
Relapse YES	21	6	15	
%		18,75	18,98734177	0.9777
Relapse NO	90	26	64	
%		81,25	81,01265823	0.9768
Toxicity YES	54	11	43	
%		34,375	54,43037975	0.0566
Toxicity NO	57	21	36	
%		65,625	45,56962025	0.0566
Relap e/o Toxic YES				
%				
Relap e/o Toxic NO				
%				
Relap + Toxic	12	2	10	
%		6,25	12,65822785	0.3269

Relapse YES	21	5	16	
%		26,31578947	17,39130435	0.3680
Relapse NO	90			
%				
Toxicity YES	54	7	47	
%		36,84210526	51,08695652	0.2602
Toxicity NO	57			
%				

Whole ALL (n° 111)	n°	PALB2 (+)	PALB2 (-)	*p-value*
		16	95	
Relapse YES	21	4	17	
%		25	17,89473684	0.5039
Relapse NO	90			
%				
Toxicity YES	54	6	48	
%		37,5	50,52631579	0.3370
Toxicity NO	57			
%				

Whole ALL (tot 111)	n°	NBS1 + e/o FANCD2 + e/o PALB2 +	wt	*p-value*
		48	63	
Relapse YES	21	9	12	
%		18,75	19,04761905	0.9685
Relapse NO	90			
%				
Toxicity YES	54	17	37	
%		35,41666667	58,73015873	0.0154
Toxicity NO	57			
%				
Relapse+Toxicity YES	12	5	7	
%		10,41666667	11,11111111	0.9075

Whole ALL (tot 111)	n°	FANCD2 + and PALB2 +	FANCD+ e/o PALB2+ e/o wt	*p-value*
		13	98	
Relapse YES	21	4	17	
%		30,76923077	17,34693878	0.2478
Relapse NO	90			
%				
Toxicity YES	54	5	49	
%		38,46153846	50	0.4362
Toxicity NO	57			
%				
Relapse+Toxicity YES	12	3	9	
%		23,07692308	9,183673469	0.1313

Whole ALL (tot 111)	n°	FANCD2 + and PALB2 +	FANCD2+ only	PALB2+ only	wt for FANCD2 and PALB2	*p-value*
		13	6	3	89	
Relapse YES	21	4	1	0	16	
%		30,76923077			17,97752809	0.2802
Relapse NO						
%						
Toxicity YES	54					
%						
Toxicity NO						
%						
Relapse+Toxicity YES	12	3	1	0	8	
%		23,07692308	16,66666667	0	8,988764045	0.1280

4.6. Discussion

To date, our study is the first report investigating the role of FA pathway in biological subgroups of paediatric ALL. Collectively, considering our analysis on 111 cases with ALL, we detected a BRCA1-*high* profile in 49 cases at diagnosis (44 %) and in remission 23 (21%); a BRCA2-*high* profile in 40 cases at diagnosis (36 %) and 20 in remission (18%); a *NBS*1 missense variant mutation (G>C;Glu185Gln) at dx/rem samples in 32 cases (28,8 %); a *FANCD*2 mutation in 19 cases at diagnosis (17%) and 1 in remission as germline aberration; a *PALB*2 mutation in 16 cases at diagnosis (14,4%). However, our findings are heterogeneously distributed among the biologic subgroups of ALL, assuming a different impact. We highlight the incidence of *NSB*1 missense variant mutation (33% vs 66%) in t(12;21) positive ALL; the rates of *FANCD*2 (18% vs 81%) and *PALB*2 (12% vs 87%) mutations in the "B-others" ALL subgroup. Moreover it is surprisingly the lack of both *FANCD*2 and *PALB*2 mutations in the t(12;21) positive ALL subset. All the information regarding results are collected in the **Table 9**.

An altered Fanconi anaemia (FA) pathway (also known as the FA–BRCA pathway) causes genomic instability and cancer in humans (*Nalepa 2018*). Genetic defects responsible for almost all FA cases have been dissected: inherited mutations in any one of the FA genes cause genetically distinct subtypes of FA. Germline inactivation of a single allele of some FA genes (such as *BRCA*1 and *BRCA*2) causes common familial breast cancer predisposition syndromes (*Nalepa 2018*). Somatic mutations and epigenetic silencing of FA genes occur in malignancies in individuals who do not have FA (*Tischkowitz 2004; Hess 2008*). Mutations and copy number variations (CNVs) of FA genes occur in a variety of cancers (*Gao J 2013*). Components of this pathway have a different role in the constitution of nuclear foci regarding the DNA repair intervention (*Kim 2012*) .

Germline mutations in *BRCA*1 and *BRCA*2 increase the susceptibility to develop breast and ovarian cancers as well as increase the risk of some other cancers (*Stieglitz* 2013, *Howlett 2002*). Recently it has been observed an increased risk of leukaemia in women with a *BRCA*2 mutation, who receive chemotherapy for breast cancer (*Iqbal 2016*).

Since mutations in *BRCA*1 and *BRCA*2 are relatively rare in the general population, nowadays most researchers focused on BRCA1 expression downregulation and/or epigenetic inactivation in sporadic tumours as a prognostic tool for chemotherapy response in patients (*De Luca 2016*). Moreover, a putative defect of DNA repair processes has been proposed as causative of early relapse in childhood ALL (*Spinella 2018*). Based on that, we focused on characterizing the expression of *BRCA*1 and *BRCA*2 among different biological subtypes of childhood ALL. In the diagnostic samples we observed a statistically significant higher expression of both *BRCA*1 and *BRCA*2 among all biological subgroups comparing with healthy donors. The significance of this phenomenon needs to be elucidated, considering it as an expression of or a reaction to genomic instability. Therefore, we observed a still high expression of both BRCA1 and BRCA2 in remission sample: this phenomenon could be considered as a response to chemotherapy from the normal cellular counterpart in the bone marrow (*Portich 2017*). BRCA1 and BRCA2 act in association with several proteins and the lack of functions of this complex confers to the cell a “BRCAness” phenotype (*Nalepa 2018*). For this reason, we focused our research on other partners as *FANCD*2, *PALB*2 and *NBS*1.

FANCD2 up-regulation supports the survival of BRCA1-deficient and BRCA2-deficient tumors by enabling error-prone S-phase cell cycle progression and replication fork stabilization through recruitment of alternative DNA damage proteins, such as translesion DNA polymerase (*Kais 2016*). *PALB*2 (as 'partner and localizer of BRCA2') was identified as a nuclear partner of BRCA2 (*Xia 2006*). PALB2 co-localizes with BRCA2, promoting its localization and stability in key nuclear structures, which in turn facilitates BRCA2 functions in DNA repair.

Activated FANCD2 (FANCD2-L) is translocated to DNA repair foci, where it co-localizes with various proteins involved in the DNA damage response, including BRCA2. Given the intimate functional links between PALB2 and BRCA2 and the similar phenotypes associated with biallelic mutations in the genes that encode them, it is plausible that monoallelic *PALB2* mutations confer susceptibility to cancer (*Fradet-Turcotte 2016*).

In particular, the N-terminal region of BRCA2 is associated with the PALB2/FANCN, which physically links BRCA1 to BRCA2 in a cell cycle-dependent manner. Mutations in either *BRCA2* or *PALB2* that disrupt this interaction result in a drastic reduction of double strand breakage (DSB) repair (*Fradet-Turcotte 2016*).

Interestingly, we found a germline mutation of FANCD2 in a case with a t(9;22) positive ALL (CT68) who showed a very aggressive disease, early detected in Guthrie card as well as in his twin (case CT69) (*Cazzaniga 2012*). Surprisingly, the FANCD2 deletion detected at germline level (Δ2), resulted different from the deletion identified in the leukemic cells (Δ35-32). The role of FANCD2 in leukaemia is yet to be clarified: recent studies suggest that the mTOR kinase inhibitor can enhance antitumor activity of conventional chemotherapy *in vitro* and *in vivo* by suppressing FANCD2 and consequently augmenting DNA damage leading to apoptosis (*Guo 2014*). This finding implies that inhibition of the FA pathway coupled with chemotherapy may be useful for the cancer treatment.

Furthermore, we investigate the status of *NBS*1 gene, which encodes the nibrin protein, forming a triheterometric complex with MRE11 and RAD50 (the MRN complex). The complex is a primary sensor of DSB (*Pastorczak 2016*). Based on previously reported evidence regarding the association between ALL occurrence and the allele C detection (*Jiang L 2011*), we decided to evaluate one of the most common NBS1 polymorphisms: the E185Q (8360 G>C Glu18Gln, rs1805794). We found a surprisingly higher incidence (almost 50%) of this polymorphism among patients with the t(12;21) positive ALL, in association with a complete lack of *FANCD*2 and *PALB*2 aberrations. Nijmegen

breakage syndrome (NBS) is an autosomal recessive chromosomal instability disorder. Clinically it is characterized by a microcephaly, immunodeficiency and a high incidence of paediatric malignancies (*Pastorczak 2016*). During anticancer therapy, these children are at high risk of life-threatening therapy related toxicity, including severe infections (*Pastoracks 2016*).

Although in our report none was affected by NBS syndrome, our findings need to be correlated with clinical course in order to understand whether this polymorphism has a role in regulating the activity of immune system in children affected by ALL, considering that the pathogenesis of this disease is tightly related to infections' response in early life (*Greaves M 2018*). We also identified in a case with t(1;19) a new *NBS*1 polymorphism characterized by a deletion at germline level (see **Table 9**).

Moreover, we highlight a single case (CT43), among the "B-others" subgroup, who presented at diagnosis with an *NBS*1-rs1805794 G>C polymorphism, a *FANCD*2 exon-1 duplication and a *PALB*2 exon-12 duplication (see Table 4), respectively. These findings could be likely related to the occurrence of the early ALL relapse, as recently reported (*Spinella 2018*). In fact, mutations of specific DNA repair genes (ATM, BRCA1, MSH2, PMS2) were detected among cases who presented an early ALL relapse (*Spinella 2018*). The authors hypothesized that the higher incidence (68% vs 18% in late-relapse group) would represent a defect in the DNA repair process which might explain the fast-evolutionary adaptability of early cases (*Spinella 2018*).

In conclusion, we here reported a complete analysis of DNA repair genes among children with different biological subgroup of ALL. Our findings strongly suggest that these genes are involved in the process of leukemogenesis and they might have a role in the occurrence of severe adverse events as life-threatening infections. To confirm this latter purpose, a larger population study is warranted.

Table 9. *NBS1, FANCD2, PALB2* alterations and *BRCA1, BRCA2* expression at diagnosis and remission in all cases B-ALL.

		ANALISI GENETICA									
B-ALL		NBS1		FANCD2		PALB2		BRCA1		BRCA2	
	CT	DIAGNOSIS	REMISSION	DIAGNOSIS	REMISSION	DIAGNOSIS	REMISSION	DIAGNOSIS	REMISSION	DIAGNOSIS	REMISSION
ALL_t(1;19)	1	rs758830069_deletion_119	rs758830069_deletion_119	wt	not analyzed	wt	not analyzed	low	low	low	low
	2	wt	not analyzed	Δ_35	wt	Exon_1_Duplication	wt	high	low	high	high
	3	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	high	high
	4	wt	not analyzed	Exon_1_Duplication	wt	Exon_12_Duplication	wt	high	low	low	low
	5	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	high	high	high	low
	6	wt	not analyzed	Exon_1_Duplication	wt	Exons_7_9_10_12_Duplication	wt	high	high	high	low
	7	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	low	low
	8	rs1805794 SNP_G>C	rs1805794 SNP_G>C	Exon_1_Duplication	wt	wt	not analyzed	high	high	low	low
	9	wt	not analyzed	wt	not analyzed	Δ_1	wt	low	low	high	low
	10	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	wt	high	low	low	low
	11	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	high	low	low
	12	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	13	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	low	low
	14	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	high	low
	15	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	low
	16	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	high	low
ALL_t(12;21)	17	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	high
	18	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	high	low
	19	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	high	low
	20	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	low	low
	21	rs1805794 SNP_G>C	not analyzed	Δ29/35	wt	Δ2-3	wt	high	low	low	low
	22	rs1805794 SNP_G>C	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	23	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	low	low
	24	wt	not analyzed	Δ1	wt	Δ10	wt	high	high	low	low
	25	rs1805794 SNP_G>C	not analyzed	wt	not analyzed	wt	not analyzed	high	low	low	low
	26	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	low	low
	27	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	high
	28	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	low
	29	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	low	low
	30	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	31	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	high	low	low
	32	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	low
	33	wt	not analyzed	Δ1	wt	Δ10	wt	low	low	low	low
	34	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	high	low	low
	35	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	high
	36	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	high	low	low	low
	37	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	high	low
	38	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	low	low
	39	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	low	low
	40	rs1805794 SNP_G>C	rs1805794 SNP_G>C	Δ1	wt	Δ6-10	wt	low	low	low	low
	41	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	low	low
	42	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	43	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	high	low	low
	44	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	45	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	high
	46	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	low	low
	47	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	low	low
	48	wt	not analyzed	Δ29	wt	Δ35	wt	high	low	low	low
	49	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	50	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	high	high	high	high
	51	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	low
	52	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	high
	53	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	high	low	low
	54	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	low	low
	55	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	low	low
	56	rs1805794 SNP_G>C	rs1805794 SNP_G>C	Δ1	wt	wt	not analyzed	high	low	low	low
	57	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	58	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	59	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	low	low
	60	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	low
	61	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	62	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	low
	63	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	64	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
B-ALL_t(9;22)	65	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	high	low	high
	66	wt	not analyzed	Δ35-32	Δ2	Δ2-6	wt	low	high	low	high
	67	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	68	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	high	low	high	high
	69	wt	not analyzed	Δ10	wt	wt	not analyzed	high	high	low	high
	70	wt	not analyzed	Δ38	wt	Δ2	wt	high	low	high	low
	71	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	high	high	low
	72	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	high	low
	73	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	high	low	high	low
OTHERS-ALL	74	wt	not analyzed	wt	not analyzed	Exon_2_Duplication	wt	low	high	high	low
	75	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	76	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	high
	77	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	high	low	low	low
	78	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	high	high
	79	wt	not analyzed	Exons 2_29_Duplication; Δ_9_30	wt	Δ_6_8	wt	high	not analyzed	high	not analyzed
	80	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	low	low
	81	wt	not analyzed	Exons_1_2_Duplication	wt	Exons_12 Duplication; Δ_6	wt	high	high	high	low
	82	rs1805794 SNP_G>C	rs1805794 SNP_G>C	Exon_1_Duplication	wt	Exon_12_Duplication	wt	high	high	low	low
	83	rs1805794 SNP_G>C	rs1805794 SNP_G>C	Exon_1_Duplication	wt	wt	not analyzed	high	high	low	low
	84	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	high	high	high
	85	wt	not analyzed	Exon 10_Duplication	wt	wt	not analyzed	low	low	low	high
	86	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	low	low
	87	wt	not analyzed	Exon 10_Duplication	wt	wt	not analyzed	high	high	low	low
	88	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	high
	89	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	high	low
	90	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	high	low
	91	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	low	low
	92	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	93	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	low	low
	94	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	95	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	low	low
	96	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	low
	97	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	98	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	99	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	high	low
	100	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	low	low	low
	101	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	102	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	high	low
	103	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	104	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
	105	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	high	low
	106	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	low	low	low
MLL_pos	107	wt	not analyzed	wt	not analyzed	wt	not analyzed	high	low	high	high
	108	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	low	high	low	high
	109	wt	not analyzed	wt	not analyzed	Exon_1_Duplication	wt	low	low	low	low
	110	rs1805794 SNP_G>C	rs1805794 SNP_G>C	wt	not analyzed	wt	not analyzed	high	high	high	low
	111	wt	not analyzed	wt	not analyzed	wt	not analyzed	low	high	low	high

Chapter 5. General discussion and conclusions

Our comprehensive study yields valuable insights into the interplay of genetic factors and treatment outcomes in pediatric B-cell acute lymphoblastic leukemia (B-ALL). The key findings point to significant relationships between genetic polymorphisms and patient responses to chemotherapy, which may ultimately influence the course of treatment.

First and foremost, our study firmly establishes the protective role of the NBS1 gene polymorphism (rs1805794 SNP_G>C) in the context of severe toxicity. By reducing the incidence of high-grade toxic episodes, this genetic variation emerges as a crucial determinant in the journey of pediatric B-ALL patients undergoing chemotherapy. These findings underscore the potential for tailoring treatment strategies based on the genetic mutations of individual patients, a promising step toward personalized medicine.

Intriguingly, our investigation also delves into the expression levels of the BRCA1 and BRCA2 genes within leukemic cells. It unravels a somewhat paradoxical relationship, as high expression of both BRCA1 and BRCA2 genes is associated with an increased incidence of severe toxicity. This observation calls for deeper exploration. It prompts us to ponder whether the enhanced gene expression may serve as a double-edged sword, offering greater sensitivity to chemotherapy agents but also intensifying side effects. The nuances of these effects need to be dissected further to unravel the intricacies of genetic interactions in the context of chemotherapy response.

Moreover, we turn our attention to the expression of BRCA1 and BRCA2 genes in host cells, particularly during the critical remission phase. Here, an intriguing correlation surfaces, indicating a higher risk of relapse in patients with elevated levels of BRCA1 and BRCA2 genes. While this association does not reach statistical significance, it warrants close scrutiny. This finding underscores the complexity of the interplay between genetic factors and the course of B-ALL. It highlights the significance of post-remission monitoring and its potential to guide prognosis.

It is important to acknowledge that our study, while robust and informative, is not without limitations.

While it involved a substantial cohort of children with B-ALL, the prospect of a more extensive, prospective project looms on the horizon. A larger population would facilitate more comprehensive analysis and validation of our findings. It could also help unveil additional genetic factors and interactions that impact treatment outcomes.

In conclusion, our study represents a significant stride in the journey toward precision medicine in pediatric B-ALL. It highlights the critical role of genetic polymorphisms, such as the NBS1 gene variation, in shaping the response to chemotherapy. It also calls attention to the multifaceted relationships between the expression of BRCA1 and BRCA2 genes and treatment outcomes. This knowledge, while valuable, is but a stepping stone toward a deeper understanding of the genetic landscape of B-ALL. It beckons for further exploration, prospective studies, and the collaborative efforts of the scientific community to unlock the full potential of personalized therapy in the realm of pediatric leukemia.

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