PhD Program in Translational and
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Childhood Acute Lymphoblastic Leukemia and genetic syndromes: unraveling know conditions and revealing novel associations

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1. General Introduction

1.1 Introduction to Acute Lymphoblastic Leukemia

Acute Lymphoblastic Leukemia: epidemiological insights

Acute Lymphoblastic Leukemia (ALL) is the most common childhood cancer. It is a clonal malignancy in which hematopoietic stem cells of the bone marrow lose their ability to differentiate into mature B or T lymphocytes.[1-4] The abnormal hematopoietic progenitors (blasts) retain an uncontrolled capacity for self-renewal and suppress other lineages of the hematopoietic system. In the Caucasian population, approximately 80% of ALL derive from abnormal proliferation of B-cell progenitors (BCP-ALL)[2], while the remaining 20% involve T-cell precursors (TCP-ALL)[1].

The peak incidence is between the third and fifth year of life, with a slight male predominance (55% males versus 45% females).8,9 In Italy, there are about 400 newly diagnosed pediatric ALL cases annually and about 15-25% of adult leukemias[4, 5].

In recent decades, significant improvements have been achieved in the treatment of childhood leukemias ALL, with the current overall survival rate (OS) in the first complete remission being 85-90% thanks to the development of international multi-agent chemotherapy protocols[1, 4-6]. Despite progress, ALL remains the leading cause of death in childhood (10% of cases), and the prognosis for children who relapse remains poor, particularly in the case of early relapse in a high-risk genetic group[7].

Pathogenesis

The etiology of ALL is still to be unraveled. It is thought to have a multifactorial origin, involving exogenous factors such as infections or ionizing radiation, together with individual genetic susceptibility [4, 5]

In recent decades, it has been demonstrated that some known cancer-predisposing conditions as well as germline variants in leukemia-related genes may predispose to ALL (see next paragraphs).

In addition to this predisposing milieu, postnatal events are required to trigger the process of leukemogenesis: somatic genomic lesions and changes in the epigenome alter gene expression and contribute significantly to leukemic transformation (Fig. 1) [8]

ARID5B	ETV6-RUNX1	Lymphoid development:
IKZF1	BCR-ABL1	PAX5, IKZF1, EBF1
CDKN2A	TCF3-PBX1	Cell cycle:
CEBPE	MLL rearrangements	CDKN2A, TP53
PIP4K2A-BM1	CRLF2 rearrangements	Transcription regulators:
GATA3	Rearrangements associated with	JAK, RAS, ERG
PAX5	BCR-ABL1-like ALL	Epigenetic changes:
TP53	(EBF1-PDGFR etc.)	CREBBP, NR3C1
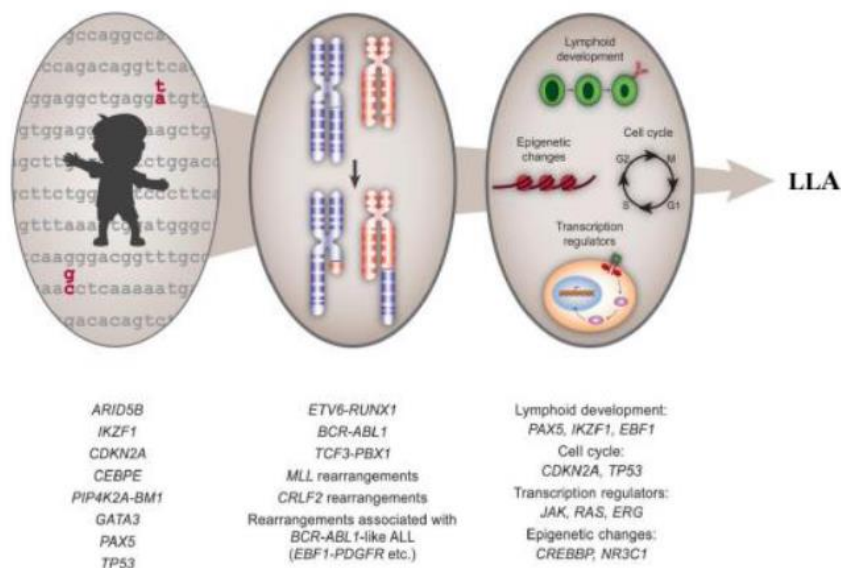
Figure 1: Visual representation of multifactorial origin of ALL

Adapted from Bhojwani et al., 2015[8]

Based on the observation that the disease is more common in childhood and more prevalent in more modern and industrialized societies, two major pathogenetic hypotheses were proposed by Kinlen[9] and Greaves[10]. Both are based on the concept that the development of ALL is indirectly promoted by an abnormal and deregulated immune response to frequent infections. Kinlen's "population mixing" model assumes that the occurrence of leukemia in a population inadequately immunized because of loss of herd immunity may be due to contact with low pathogenic infectious agents imported from abroad[9]. In contrast, Greaves' pathogenetic theory is based on a two-stage model: individuals who are predisposed because they acquired a preleukemic clone prenatally and did not have early exposure to pathogens responsible for common infections are more likely than others to develop acute leukemia because of the abnormal response of the immune system triggered by later and delayed exposure to common microorganisms[10]. Recently, Combo-de-Oliveira and colleagues highlighted some of the strongest risk factors for the development of ALL, such as cesarean section and birth order or lack of breastfeeding (Fig. 2)[11]. These findings are consistent with the "delayed infection hypothesis," which suggests that infants who are isolated from infections early in life may be more susceptible to ALL when exposed to infections later in childhood. [8, 10, 12, 13]

Genetic alterations	Delivery mode	CMV	Birth order	Cytokine abnormalities	Breastfeeding	Daycare exposure	Childhood infections
ETV6-RUNX1 *IKZF1* *PAX5*	C-section	CMV+	First born	↓IL10 ↑Arg II	Breastfed	Daycare attendance	↑Infections
↑ALL	↑ALL	↑ALL	↑ALL	↑ALL	↓ALL	↓ALL	↑ALL

Germline	Perinatal	Postnatal

Figure 2: Individual and exogenous risk factors that may contribute to the development of ALL. Adapted from Wiemels J and Gallant R, 2022[13]

In addition, retrospective studies searching for leukemia-associated fusion genes, hyperdiploidy, and clonotypic rearrangements of immunoglobulins (Ig) and the T-cell receptor (TCR) in the blood of newborns and identical twins have confirmed the prenatal origin of some forms of childhood leukemia[10, 12, 14, 15]. However, the heterogeneity of presentation, the low concordance (10%) in genetically identical twins, and the appearance of the disease in adulthood support the view that postnatal events are necessary for the onset of the disease. Therefore, as in other neoplasms, the accumulation of genetic alterations (balanced translocations, point mutations, over-expression, and downregulation of genes due to epigenetic changes) involved in the regulation of transcription and translation of proteins with oncogenic and tumor suppressive functions can be assumed to be responsible for the cancerous transformation of lymphoid stem cells[10, 12, 14, 15].

Genetics of BCP Acute Lymphoblastic Leukemia

BCP-ALL includes several subtypes, each characterized by different genetic alterations and by different patterns of gene expression [1, 2, 16] Within B- ALL subtypes, there are significant differences in initial genetic abnormalities, secondary genetic alterations, and prognosis. In addition, there is a growing understanding of how inherited genetic variations, in both coding and non-coding regions, may predispose individuals to ALL [17]. In the majority of B- ALL subtypes, secondary genomic alterations play a critical role in the development of leukemia and also influence the likelihood of relapse (Fig. 3) [18]
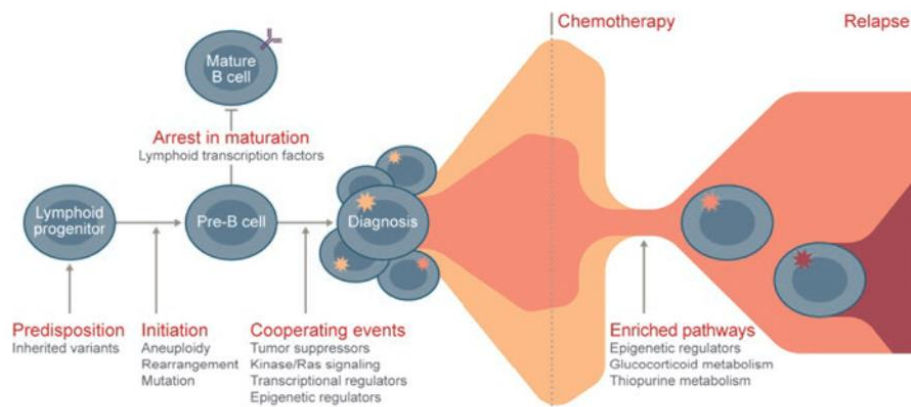
Figure 3: Schematic representation of the different genetic alterations that contribute to leukemogenesis and risk of relapse.
Adapted from Roberts K and Mullighan C, Springer Nature, 2022

Genetic categorization of BCP-ALL was initially performed by cytogenetic karyotyping, targeted fluorescence in situ hybridization (FISH), and standard molecular assays that were able to identify [19] abnormal chromosomes numbers (high hyperdiploidy and hypodiploidy) as well as fusion genes such as *ETV6-RUNX1, BCR-ABL1*, and *TCF3-PBX1* and rearrangements of *KMT2A* (MLL), which were present in approximately two-thirds of ALL cases in childhood. [5]

The introduction of microarray profiling for gene expression revealed that known subtypes of BCP- ALL have distinct gene expression patterns. [20]. SNP arrays specifically pointed out recurrent DNA copy number alterations (CNA), especially in genes involved in lymphoid development (*PAX5, IKZF1, EBF1*) [21]. Transcriptome sequencing (RNA-seq) has proven to be a powerful tool to understand the molecular classification of B- ALL and identify genetic influencing factors. While RNA-seq couldn't capture all sequence and structural changes, it provided extensive data on gene expression, gene rearrangements, chromosomal aneuploidy, and mutations. RNA-seq was fundamental, for instance, for the identification of Ph-like (BCR-ABL1-like) ALL [22-24].

In recent years, new recurrent rearrangements (e.g., *DUX4*, *MEF2D*, and *ZNF384*) have been associated with distinct gene expression profiles [25, 26]. Large-scale B-ALL RNA -seq studies involving nearly 2000 samples led to further findings, including less common subtypes caused by chromosomal rearrangements (e.g., *NUTM1* and *BCL2MYC/BCL6*), subtypes caused by sequence mutations (e.g.., *PAX5*

P80R and *IKZF1* N159Y), and subtypes characterized by differential gene expression but different alterations in a single gene (PAX5alt, with fusions, sequence mutations, and intragenic amplifications of the DNA-binding transcription factor) [26, 27] (Fig. 4; Fig. 5))
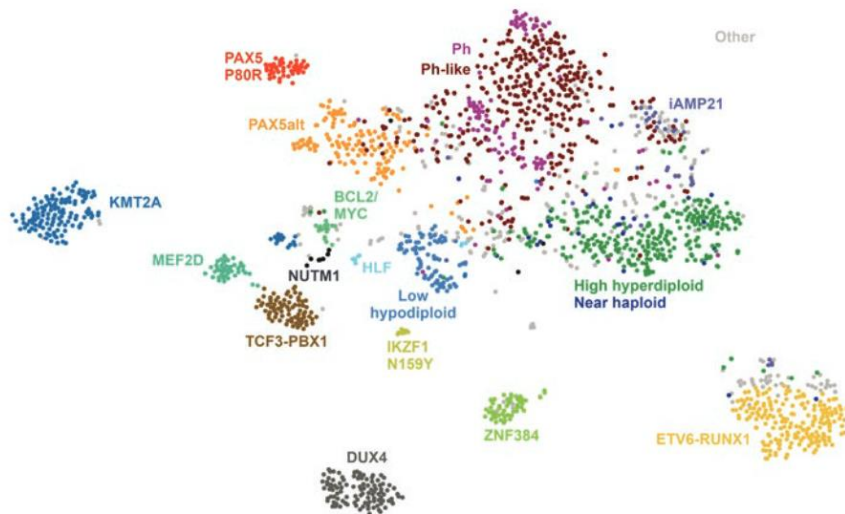
Figure 4: Clustering of different subtypes of ALL, based on gene expression profiling Adapted from Roberts K and Mullighan C, 2022
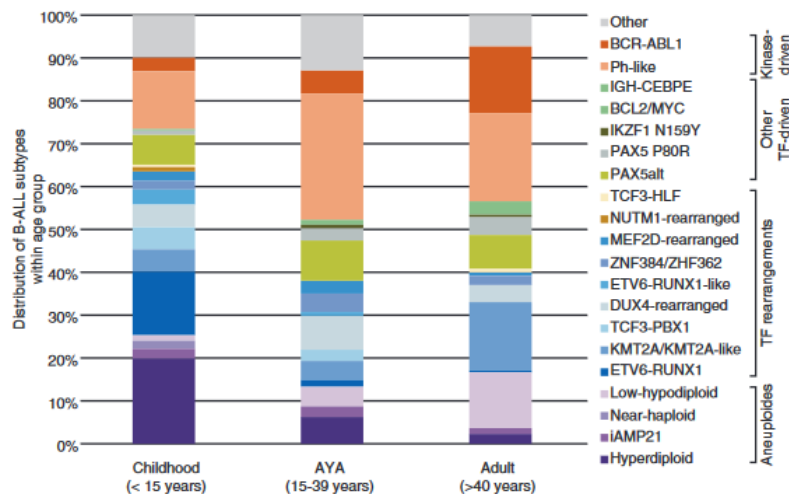
Figure 5: Distribution of different BCP-ALL genetic subtypes comparing different ages: childhood, Adolescence and Young Adults (AYA) and Adult Adapted from Adapted from Roberts K and Mullighan C, 2022

In the next two paragraphs, we will focus on two specific subtypes of BCP-ALL that are relevant to this thesis project.

Hypodiploid pediatric ALL

As mentioned earlier, hypodiploidy is defined as the presence of less than diploid number of chromosomes in the blast cells (< 46 chromosomes; DNA index < 1.0). At ALL, 30-40% of patients have a hyperdiploid karyotype, while only 1% have a hypodiploid karyotype[28]. Hypodiploid ALL can be divided into three different subgroups: high-hypodiploid (40-45 chromosomes), low-hypodiploid (31-39 chromosomes; DNA index < 0.8), and near-haploid (24- 30 chromosomes)[28-30]. Interestingly, and complicating the picture, it has been demonstrated that the hypodiploid genome can undergo reduplication leading to a hyperdiploid karyotype ("masked" hypodiploid ALL): This can be revealed by traditional testing methods such as DI, karyotype and FISH, which can distinguish the hypodiploid clone and the duplicated hyperdiploid clone [31].

Nearly haploid and low-hypodiploid patients share a common/pre-B immunophenotype and usually have low white blood cell counts at diagnosis; in contrast, high-hypodiploid patients also have a T-lineage immunophenotype [29]. Near-haploid ALL patients are younger than

low-hypodiploid ALL patients (> 10 years old, median 13 years) at the time of diagnosis; an equal incidence in males and females has been reported [32].

Although the overall prognosis of ALL has improved in recent decades, hypodiploid ALL patients still have a poor outcome[32-34]. In a large study published in 2019, Pui et al. found that the 8-year event-free survival rate (EFS) for the entire hypodiploid cohort was 57.5%; only the highly hypodiploid ALL patients showed a better outcome with an 8-year rate EFS of 73.7%[34]. The only known prognostic indicator for hypodiploid ALL patients is minimal residual disease (MRD), and treatment (chemotherapy) or hematopoietic stem cell transplantation (HSCT) is based on MRD stratification [35].

Nevertheless, there are few solid data on the efficacy of HSCT in these patients, and further research is needed on different treatment strategies for these categories of patients[34].

Chromosomal patterns and mutation profiles in hypodiploid ALL

Low-hypodiploid and near-haploid ALL show a specific pattern of conserved disomies: Chromosomes X/Y, 8, 10, 14, 18, and 21 are preserved in near-haploid ALL, whereas X/Y, 1, 5, 6, 8, 10, 11, 14, 18, 19, 21, and 22 are preserved in low-hypodiploid ALL. Of note, the absence of chromosome 21 aneuploidy is never observed[29, 30]. In addition, these two hypodiploid subtypes have recently been shown to have distinct mutational profiles. Near-haploids harbor somatic genetic variants in genes such as *NF1, CREBBP, CDKN2A/B*, histone gene cluster 6p22, *IKZF3*, and *PAG1*. In contrast, mutations in *IKZF1*, *RB*, and *TP53* are characteristic of low-hypodiploid cells. The latter gene is found mutated in more than 50% of cases [32]. Although *TP53* is one of the most common somatic genetic aberrations in cancer, it is quite rare in ALL. There are only two cases where *TP53* is altered in ALL: low-hypodiploid and recurrent ALL [28, 32]).

In 2015, Mullighan and colleagues reported mutations in *TP53* in more than 90% of low-hypodiploid ALL cases. Interestingly, they also found that 40% of these patients carried a germline variant of *TP53*, making the disease one of the possible manifestations of Li-Fraumeni syndrome (LFS) [32-35].

PAX5-Driven BCP-ALL

The transcription factor PAX5, known for its role in controlling B-cell lineage formation and differentiation, plays an important role as an

initiating or contributing factor in the pathogenesis of BCP-ALL. These PAX5-related changes can be categorized as follows:

1.   Disease-causing alterations: these include *PAX5* rearrangements leading to chimeric fusion oncoproteins and the P80R mutation [5, 16, 159, 160, 161]. In addition, there are rearrangements or focal intragenic amplifications in PAX5-altered (PAX5alt) ALL[27, 36-39].

2.   Secondary lesions: these are focal *PAX5* deletions found in 30% of ETV6-RUNX1 ALL and *PAX5* mutations observed in multiple subtypes[38].

3.   Germline alterations: these genetic alterations predispose individuals to ALL [40-44].

In mouse models, Pax5 heterozygosity has been shown to interact with constitutive activation of the JAK-STAT pathway and contribute to the development of BCP-ALL. This supports the notion that Pax5 functions as a haploinsufficient tumor suppressor [45].

PAX5alt represents a subtype of BCP-ALL characterized by similar gene expression profiles in leukemic cells but with a broad spectrum of underlying PAX5 alterations. These alterations include cases with different PAX5 rearrangements in which the N-terminal DNA-binding domain is usually retained but the C-terminal transactivation domain is lost. In other cases, there is focal intragenic amplification of the PAX5 DNA-binding paired domain (PAX5amp) or sequence mutations.

Specific alterations within this subgroup are associated with different gene expression profiles. PAX5alt is most common in children and young adults (AYA population) and is associated with intermediate outcomes[27].

The PAX5-P80R subtype is characterized by the presence of the PAX5-P80R mutation, which is often associated with inactivation of the wild-type PAX5 allele by deletion, loss-of-function mutation, or copy-neutral loss of heterozygosity [5, 159, 160]. Importantly, heterozygous Pax5P80R/+ knock-in mice develop transplantable BCP-ALL with genetic inactivation of the wild-type Pax5 allele [27]. The prevalence of *PAX5* P80R increases with age and is associated with an intermediate to favorable prognosis [27, 36]. Other notable cooperating lesions include structural rearrangements of chromosomal arms 9p and 20q, particularly in association with the presence of dic(9:20). In addition, mutations in members of the RAS and JAK-STAT pathways are particularly common, highlighting the potential for targeted therapeutic intervention.

Genetics of T Acute Lymphoblastic Leukemia

T-cell acute lymphoblastic leukemia (T-ALL) accounts for 10–15% of pediatric cases of acute lymphoblastic leukemia [46]. It typically manifests at a later age (around 9 years) compared to BCP-ALL and is often diagnosed in adolescence. The distribution between males and females shows a significantly higher prevalence in males, especially in patients younger than 40 years. At diagnosis, T-ALL patients usually

have elevated white blood cell counts in the peripheral blood and present with symptoms due to infiltration of immature cells in the bone marrow (resulting in cytopenias) and lymphoid organs (manifested by enlargement of lymph nodes, splenomegaly, and thymic masses in the chest) [47, 48].

T-ALL exhibits considerable heterogeneity in terms of transcriptional profiles, genetics, immunophenotypes, and clinical characteristics. They are classified into different clinical biological groups based on their gene expression patterns, which correspond to the developmental stage at which thymocyte development is arrested [47]. Among these groups, early T-cell precursor leukemias (ETPs) are characterized by early blockage of thymocyte development, especially at the CD4-CD8 double-negative stage. They are characterized by the expression of genes associated with hematopoietic stem cells and myeloid progenitors [49]. In contrast, typical T-ALL cases have gene expression profiles more closely associated with maturation stages of thymic populations [47].

Transformation of T-ALL is a carefully coordinated process involving mutations that interact through convergent and complementary mechanisms. These mutations are associated with distinct oncogenic pathways and molecular subgroups.

ETP ALLs, for example, have a lower frequency of *NOTCH1* mutations and *CDKN2A* deletions. Instead, they have a higher frequency of mutations in signaling factors such as NRAS and FLT3, which are

common in myeloid leukemias. In addition, mutations in epigenetic regulators such as *EZH2*, *IDH1*, *IDH2*, and DNMT3A are more common in ETP-T ALLs. In addition, these ETP-T-*ALLs* have mutations in transcription factors that play key roles in hematopoietic and T-cell development, such as RUNX1, GATA3, and ETV6 [47, 50].

On the other hand, typical T-ALLs characterized by an early cortical immunophenotype (CD1a, CD4, CD8 positive) are associated with the activation of transcription factor oncogenes such as TLX1, TLX3, NKX2.1 and NKX2.2. They also have a very high prevalence of NOTCH1 mutations and CDKN2A deletions. In addition, they are associated with mutations in genes such as *PHF6, BCL11B, WT1* and *NUP214-ABL1* rearrangements [47]

Finally, T-ALLs with a late cortical thymocyte immunophenotype characterized by CD4, CD8, and CD3 positivity are clearly associated with alterations leading to abnormal expression of TAL1 and LMO factors. These T-ALLs also have deletions and mutations in the PTEN tumor suppressor gene [47]

1.2 Genetic predisposition to Acute Lymphoblastic Leukemia

Over the past decade, genomic analyzes have enabled major advances in our understanding of the genetic and biological basis of ALL, suggesting increasing involvement of hereditary predisposition[51, 52].

By sequencing the genomes of rare families with a predisposition to leukemia and studying large cohorts of ALL cases, researchers have uncovered a growing list of genes and associated genetic conditions that increase susceptibility to lymphoblastic leukemia[51-55].

Just as somatic genetic information is critical to understanding ALL, germline genetic data can have a significant impact on clinical care. They can help identify children who are at higher risk of developing treatment-related adverse events, second cancers, and nononcologic health problems[53, 54].

Although several syndromes have already been associated with cancer predisposition, emerging evidence shows that the prevalence and spectrum of cancer-predisposing mutations in children and adolescents have not yet been recognized.[51, 53, 54]

Genetic susceptibility to ALL: high penetrance genes.

The first reports of familial cases of ALL appeared in the literature in the early 1950s to 1960s [10–12-13]. However, it was not until germline variants in TP53 were identified as the cause of Li-Fraumeni syndrome (LFS) in 1990 [56]that the genetic basis for familial ALL came into focus.

Thus, it has become clear that there are several Mendelian genetic conditions that increase the risk of developing ALL. In most cases, these predisposing conditions follow an autosomal dominant inheritance pattern. These disorders are primarily the result of deleterious germline alterations affecting key genes involved in various cellular processes, including differentiation, proliferation, apoptosis, DNA damage repair, and intracellular signaling (Fig. 6)[53, 54, 57].

The clinical syndromes that predispose to ALL may be caused by germline variants, in particular:

-       in genes closely associated with B-lymphocyte differentiation, are primarily associated with increased risk of B-cell ALL

-       in genes with more diverse functions that predispose to a broader range of cancers and other associated manifestations
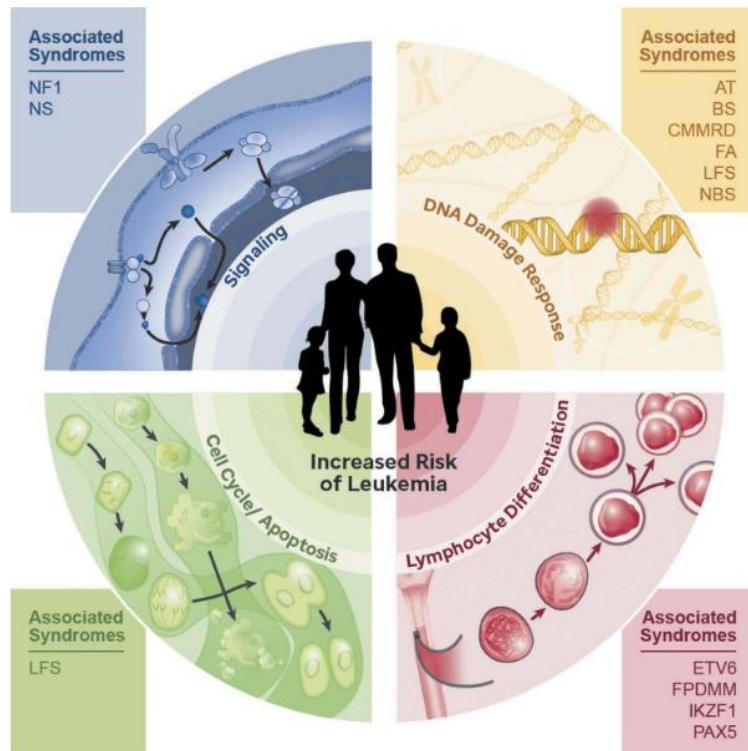
Figure 6: Cancer predisposing syndromes that predispose to both solid and hematological malignancies are caused by pathogenic variants in genes involved in critical cellular pathways.

Abbreviations: CMMRD, Constitutional mismatch repair deficiency; ETV6, ETV6-associated predisposition; FA, Fanconi anemia; FPDMM, Familial platelet disorder with predisposition to myeloid malignancy; IKZF1, IKZF1-associated predisposition to B-ALL; LFS, Li-Fraumeni syndrome; NBS, Nijmegen breakage syndrome; NF1, Neurofibromatosis type 1; NS, Noonan syndrome; PAX5, PAX5-associated leukemia predisposition.

Adapted from Bloom M et al., 2019

[53]

Syndrome predisposing to both solid and hematological malignancies, including ALL

Li-Fraumeni syndrome

Li-Fraumeni syndrome (LFS, OMIM #151623) is a cancer predisposition syndrome caused by germline mutations in *TP53*. It is associated with an increased risk for childhood- and adult-onset malignancies. The lifetime risk of developing cancer in individuals affected with LFS is ≥70% for men and ≥90% for women. The five most common cancers that occur in this condition are adrenocortical carcinomas, breast cancer, central nervous system tumors, osteosarcomas, and soft-tissue sarcomas [58] Moreover, LFS is associated with an increased risk of several other cancers including hematological malignancies , even if less frequently (accounting for only 2-4% of all cancers in LFS individuals) [59]. The types of leukemia associated with LFS encompass acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and myelodysplastic syndrome (MDS). It's worth noting that most cases of ALL in LFS individuals exhibit a 'low' hypodiploid phenotype[59, 60].

ALL in LFS tends to manifest later in childhood, with a median age of onset at 15.5 years, in contrast to 7.3 years for children without LFS. Additionally, the leukocyte counts at presentation are often lower [60]."

With the aim of early tumor detection and reduction of cancer and treatment-related morbidity and mortality, different protocols for clinical surveillance of TP53 mutation carriers have been proposed.

Among them the "Toronto protocol" was established as a surveillance strategy for patients with LFS (Villani et al., 2016). This protocol provides regular ultrasounds and physical examinations, annual magnetic resonance imaging (MRI) and dermatologic evaluation, endoscopies, and colonoscopies. A 11-years follow-up of this protocol showed that the diagnosis of tumors on the surveillance "arm" was performed in a low grade or premalignant stage of the disease, suggesting that early detection through surveillance may identify lesions before malignant transformation. Moreover, an improved overall survival (OS) was observed in individuals undergoing surveillance: 5-year OS, 88.8% in the surveillance group versus 59.6% in no surveillance group [61, 62]

Constitutional Mismatch Repair Deficiency

Constitutional mismatch repair deficiency (CMMRD) is an autosomal recessive disorder that results from homozygous or compound heterozygous mutations in mismatch repair (MMR) genes that play a critical role in correcting single-stranded DNA mutations that occur during replication. These genes include MSH2, MSH6, MLH1, PMS2 and, in rare cases, deletions in the 3'-region of EPCAM, a gene located near the MSH2 promoter[63].

Heterozygous pathogenic variants in MMR genes are responsible for Lynch syndrome, an autosomal dominant disorder associated with the development of adult cancers, particularly colon and endometrial cancers [63]. In contrast, CMMRD is associated with an exceptionally high incidence of cancer that begins in early childhood [63, 64].

Approximately one-third of CMMRD patients develop hematologic malignancies, with a median age at the diagnosis of 6 years (ranging from 0 to 21 years) [64, 65]. Non-Hodgkin's lymphoma (NHL), which is predominantly T-cell derived, is the most reported hematologic malignancy, with fewer cases of acute lymphoblastic leukemia (ALL), which is also predominantly T-cell derived[65]. Hematologic malignancies occur more frequently in individuals with *MLH1* or *MSH2* variants (38%) than in *MSH6* (25%) or *PMS2* carriers (16%)[64, 65].

Patients with CMMRD usually tolerate conventional ALL chemotherapy without excessive toxicity; however, they are prone to leukemia relapse. Most CMMRD-associated tumors already exhibit a hypermutation phenotype with more than 100 mutations per megabase of DNA[53]. This accumulation of somatic mutations in CMMRD-associated tumors, whether induced by treatment or not, likely contributes to treatment resistance.

Fanconi Anemia

Fanconi anemia (FA, OMIM 227650) is the most prevalent cause of inherited bone marrow failure[66, 67]. The underlying molecular mechanism of Fanconi anemia primarily revolves around a faulty homologous recombination DNA repair pathway. It involves defects in proteins and other enzymes responsible for repairing damaged DNA resulting from exposure to various substances like alkylating agents, radiation, and cytotoxic drugs. Fanconi anemia is frequently associated with congenital anomalies and carries a heightened risk of hematological and solid tumors[66, 67]. Among hematological

malignancies, MDS is the most common malignancy, while acute myelogenous leukemia is the second most common cancer. ALL are rare and only some cases have been reported[66, 67].

Syndromes predisposing to hematologic malignances, including ALL

Down Syndrome

Down syndrome (DS) is a genetic condition characterized by constitutional trisomy of chromosome 21 and is the most prevalent chromosomal disorder[68]. It was among the earliest genetic syndromes linked to an elevated risk of childhood leukemia, with initial reports dating back to the 1930s[69].

Children with DS have a 20-fold higher risk of developing ALL compared to children without DS and a 500-fold increased risk of developing acute megakaryoblastic leukemia (AMKL)[69, 70]. The onset of leukemia in DS occurs in two distinct peaks, with the first peak in newborns and the second occurring between 3 and 6 years of age. This increased risk may also extend into adulthood. Leukemias that develop in infants with DS are predominantly myeloid [70]. In contrast, ALL occurs in children with DS at similar ages to children without DS [71]. Most of ALL cases seen in children with DS originate from B cell precursors (only few cases of T-ALL were reported)[71]. Historically, DS-ALL has been considered to have a poorer prognosis than non-DS ALL due to resistance to therapy and/or treatment-related mortality. However, recent reports have suggested that DS patients with ALL fare

as well as those without DS when provided with appropriate supportive care[71].

Common somatic genetic abnormalities often observed in sporadic B-ALL are less frequent in DS B-ALL, such as ETV6-RUNX1 translocation and hyperdiploidy[72, 73]. Conversely, up to 60% of DS-ALL cases show overexpression of Cytokine Receptor Like Factor 2 (CRLF2), a type I cytokine receptor that binds thymic stromal lymphopoietin (TSLP) and is frequently associated with mutations activating the JAK-STAT pathway [72, 73]

Noonan syndrome

Noonan syndrome (NS, OMIM #163950) Noonan syndrome (NS) is an autosomal dominant genetic condition that arises from inherited variants affecting genes that encode components of the RAS/mitogen-activated protein kinase (MAPK) pathway [74, 75]. It accounts for 1:1000 - 1:2500 live births. Around 50% of individuals with NS carry heterozygous inherited variants in *PTPN11*, while a smaller number have alterations in genes like *SOS1, KRAS, RAF1, BRAF*, and *MEK1*, among others. NS occurs in approximately 1 in 1000 to 1 in 2500 live births[74, 75].

Hematological malignancies, such as juvenile myelomonocytic leukemia (JMML) and ALL, are the most commonly occurring cancers in individuals with NS [76, 77]. Focusing on ALL in NS patients: It occurs in 0.3–0.5% of individuals with NS with an average onset age of 6 years (ranging from 1.5 to 17 years), it exclusively belongs to the B cell

phenotype and It is associated with a germline *PTPN11* or *SOS1* variant [76].

NS patients with germline *PTPN11* variants are more prone to developing hyperdiploid ALL, and their disease typically responds well to therapy[76]. Although late complications are not frequently documented, one report indicated that one-fifth of NS patients treated for ALL subsequently developed myelodysplasia (MDS). In some cases, MDS resolved spontaneously but still exhibited persistent thrombocytopenia, while in others, it progressed into a JMML-like neoplasm or acute myeloid leukemia (AML)[77].

Neurofibromatosis type 1

Neurofibromatosis type 1 (NF1, OMIM #162200) is one of the more prevalent genetic disorders, affecting approximately 1 in every 3,000 births [78].

NF1 patients may develop both benign and malignant tumors, such as Lisch nodules (iris hamartomas), cutaneous and plexiform neurofibromas, central nervous system (CNS) tumors, and, less commonly, hematologic malignancies [78]. It has been known for decades that individuals with NF1 have an increased susceptibility to leukemia, including chronic myelomonocytic leukemia (CMML), juvenile myelomonocytic leukemia (JMML)[79], acute myeloid leukemia (AML), and, to a lesser extent, acute lymphoblastic leukemia (ALL)[78]. JMML is the most prevalent leukemia in this context, with NF1 patients facing a 200–500-fold higher risk compared to those

without NF1 [79]. A large population-based study in the United Kingdom investigating leukemia and non-Hodgkin lymphoma associated with NF1 found only 12 cases of ALL, with a relative risk of 5.4 (95% CI 2.8–9.4) [80]. Among these 12 ALL cases, three were of T-lineage origin, while nine were of B-lineage. The average age at diagnosis was 5.1 years for B-ALL (ranging from 1.3 to 12.3 years) and 11.4 years for T-ALL (ranging from 9.8 to 14.5 years)[78, 81] .

Nijmegen Breakage syndrome

Nijmegen breakage syndrome (NBS, OMIM 251260) is an inherited DNA repair disorder known for its heightened susceptibility to childhood lymphoid malignancies, among which diffuse large B-cell lymphoma (DLBCL) is the prominent subtype[82]. It is an autosomal recessive condition caused by homozygous or heterozygous compound mutation in *NBN*, that synthesizes for Nibrin which is part of complex serves that detects DNA double-strand breaks (DSB) and is essential for facilitating the efficient monomerization and autophosphorylation of ATM following DNA DSB damage. In most of the patients a truncating deletion of NBN (c.657_661del5, pK219fsX19) is identified[82].

ALL a is a much less common form of lymphoproliferative disorder in NB), with a notable predominance of the T-cell immunophenotype[83]. Interestingly, many children with NBS-associated ALL have a central nervous system involvement at the time of ALL diagnosis. Patients with NBS show an increased toxicity profile compared to other patients[83].

Ataxia Telangiectasia

Ataxia telangiectasia (A-T, OMIM 208900) is a rare autosomal recessive disorder characterized by the presence of cutaneous telangiectasias, progressive ataxia due to cerebellar atrophy, and an increased susceptibility to malignancies (especially lymphoid tumors), immune deficiencies, and radiosensitivity[84]. It is caused by homozygous or heterozygous compound variants in *ATM*, that is involved in the DNA repair mechanisms, cell cycle's regulation, and the cellular response to external stimuli like oxidative stress, ionizing radiation, and alkylating agents[85]. Approximately 25% to 30% of individuals with A-T may experience tumor development, especially T-ALL and lymphoma typically manifest during early childhood[83, 86]. In older age, A-T patients may also be at risk for solid tumors, including breast and ovarian cancer, melanomas, gastric cancer, or liver tumors[84].

Familial platelet disorder with predisposition to myeloid malignancy

Familial platelet disorder with predisposition to myeloid malignancy (FPDMM; OMIM #601399) is an autosomal dominant condition resulting from inherited variants in the *RUNX1* gene, which encodes the RUNT-related transcription factor 1 (RUNX1, previously known as CBFA2 or AML1)[87]. RUNX1 serves as a partner to Core-Binding Factor Subunit Beta (CBFB) to form a heterodimeric transcription factor. This gene is one of the most frequently mutated genes in various types of leukemias, including MDS, AML, ALL, CMML[87, 88]. RUNX1 plays a role in several leukemia-associated translocations, such as the t(12;21)

ETV6-RUNX1 in pediatric B-ALL, the t(8;21) *RUNX1T1-RUNX1* in AML, and more than 50 other translocations[87].

To date, more than 80 families with pathogenic germline *RUNX1* variants have been documented[88-90]. Individuals with FPDMM typically experience lifelong thrombocytopenia and functional platelet defects, which may or may not be associated with bleeding[88]. Those with germline *RUNX1* variants have an elevated risk of developing MDS, AML, and, to a lesser extent, T-ALL[88, 90]. Approximately 30–40% of mutation carriers eventually develop a hematologic malignancy [85,86]. To date, only 11 individuals with RUNX1 mutations have been reported to develop T-ALL, with three of them progressing to AML within five years of the initial T-ALL diagnosis[57, 90]. While somatic *RUNX1* mutations have been identified in 7% of B-ALL cases, there have been very few reported cases of individuals with FPDMM developing B-ALL[87].

Syndromes that primarily predispose to ALL

PAX5-associated leukemia predisposition
PAX5-associated leukemia predisposition (susceptibility to ALL 3; OMIM #167414) is a condition caused by germline variant in PAX5; it increases the risk of developing ALL and is inherited in an autosomal dominant manner[57].
In 2013, three unrelated families were reported in which individuals affected by ALL had the same heterozygous germline *PAX5* p.Gly183Ser missense variation[40, 41]. Interestingly, within these

families, some carriers of the *PAX5* variant did not develop ALL, suggesting that this specific genetic variation does not always result in the condition (incomplete penetrance). To date, all the ALL samples associated with germline *PAX5* variants that have been studied show a loss of the normal *PAX5* allele. This loss typically occurs through the formation of an isochromosome or a dicentric chromosome 9q[41]. Consequently, PAX5 appears to function as a classic tumor suppressor gene in this context, with germline mutations acting as the initial genetic 'hit,' and the loss of the remaining normal (wild-type) *PAX5* allele serving as the second genetic 'hit' within the leukemia cells.

ETV6-associated leukemia predisposition

ETV6-associated leukemia predisposition (thrombocytopenia V; OMIM #616216) is caused by germline mutations in *ETV6*, that encodes a transcriptional repressor belonging to the ETS family[57, 91, 92]. It plays a critical role in establishing hematopoiesis in mouse bone marrow (BM) and ensuring the survival of hematopoietic stem cells (HSCs). In 2015, germline damaging *ETV6* variants were described in eight unrelated families in which B-ALL and thrombocytopenia were inherited in an autosomal dominant manner, [57, 92]. To date, 96 individuals from 23 families have been described in this context[93]. Among these 96 individuals, 25–30% are reported to have developed leukemia, most commonly B-ALL with a hyperdiploid karyotype. There have also been rare cases of mixed-phenotype leukemia, acute or chronic myeloid leukemia, B-cell non-Hodgkin lymphoma, and multiple myeloma reported[93]. In a study involving 38 ALL patients

with germline *ETV6* variants, two individuals developed secondary myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML), suggesting a possible link to therapy-associated myeloid neoplasms[94].

Nearly every individual with a germline *ETV6* variant experiences mild to moderate thrombocytopenia, with some showing a tendency to bleed and/or platelet functional abnormalities[91]. Severe thrombocytopenia is only observed in cases associated with myelodysplastic syndrome[91]. Examination of the bone marrow in individuals with germline *ETV6* variants who do not have leukemia reveals characteristics such as megakaryocyte hyperplasia, hypolobulated megakaryocytes, mild dyserythropoiesis, and abnormal myeloid cells[91, 95].

IKZF1-associated predisposition to B-ALL (OMIM #616873)

IKZF1, which stands for IKAROS Family Zinc Finger 1, is responsible for encoding the founding member of the IKAROS family of transcription factors[96]. It is expressed in various hematopoietic cells and plays a critical role in specifying lymphoid lineages and guiding the differentiation of pro-B cells into pre-B cells. The presence of somatic IKZF1 mutations appears to be associated with a poorer response to therapy[57].

In 2018, Churchman and colleagues reported a family with five individuals who carried a heterozygous germline *IKZF1* variant (p.Asp186fs), predicted to truncate the protein within the region of the

N-terminal zinc fingers[97]. Two of these carriers developed B-ALL, suggesting that this variant may predispose individuals to leukemia[97]. To investigate further, they conducted targeted sequencing of *IKZF1* in a cohort of 4,963 pediatric ALL cases and identified 27 distinct non-silent *IKZF1* coding variants among 43 individuals (0.9%), with nearly all of them having B-ALL[97]. Genotype-phenotype studies did not reveal any obvious correlations, but several of the individuals with *IKZF1* mutations showed signs of B lymphopenia, indicating an underlying immunodeficiency.

ALL genetic susceptibility: low penetrance genes

Over the past decade, numerous genome-wide association studies (GWAS) have aimed to uncover the role of common germline variants, including coding and noncoding single nucleotide polymorphisms (SNPs), in the evolution of ALL. Previous GWAS efforts have uncovered susceptibility loci associated with pediatric ALL involving genes known to play critical roles in hematopoiesis. These genes include *IKZF1*, *ARID5B*, *CEBPE*, *GATA3*, *BMI1*, *CDKN2A[98]*. In addition, GWAS have uncovered risk loci specifically associated with drug response, relapse, and development of ALL in certain ethnic groups, which may explain ancestral differences. Recently, a GWAS by Qian and colleagues focused on 1,191 children with T- ALL and identified the first germline risk locus exclusively associated with this subtype of leukemia[98]. This risk focus includes multiple SNPs associated with *USP7* (ubiquitin-specific peptidase-7), a gene encoding a ubiquitin-specific protease

that is somatically mutated in 12% of pediatric and young adult T- ALL cases.

Susceptibility loci are summarized in Table 1.

Locus	Gene	SNP ID	Cohort	ALL Subtype
2q22.3		rs17481869	Multi-ethnic	*ETV6-RUNX1* B-ALL
3q28	*TP63*	rs17505102	European	*ETV6-RUNX1* B-ALL
7p12.2	*IKZF1*	rs11978267	European	B-ALL
7p15.3	*SP4*	rs2390536	Multi-ethnic	B-ALL
8q24.21		rs28665337	Multi-ethnic	B-ALL or T-ALL
9p21.3	*CDKN2A/B*	rs3731217, rs77728904	European	B-ALL or T-ALL
10p12.2	*PIP4K2A*	rs7088318	Multi-ethnic	B-ALL or T-ALL
10p14	*GATA3*	rs3824662	Multi-ethnic	Ph-like B-ALL
10q21.2	*ARID5B*	rs10821936	European	Hyperdiploid ALL or T-ALL
10q26.13	*LHPP*	rs35837782	European	B-ALL or T-ALL
12q23.1	*ELK3*	rs4762284	European	B-ALL
14q11.2	*CEBPE*	rs2239633	European	B-ALL
16p13.2	*USP7*	rs74010351	Multi-ethnic	T-ALL
17q12	*IKZF3*	rs2290400	Multi-ethnic	B-ALL or T-ALL
21q22.2	*ERG*	rs2836365	Multi-ethnic	ALL

Table I: List of susceptibility loci associated with childhood ALL. Abbreviations: ALL, acute lymphoblastic leukemia; B-ALL, B cell precursor ALL; T-ALL, T cell ALL Bloom M et al., 2019[53].

1.3 The Cohesin complex

Cohesin ring is an evolutionary conserved multi-protein complex. In all Eukaryotic organisms, it consists of four core subunits: two subunits SMC (*Structural Maintenance of Chromosomes*), *SMC1A*, *SMC3*, and two subunits SCC (*Sister Chromatid Cohesion*), either *STAG1* or *STAG2* and *RAD21*. The last one is also known as 'double-strand-break repair protein'[99]. (Fig.7)

SMC1 and SMC3 interact with each other creating a heterodimer, instead RAD21 bridges both SMC subunits by binding SMC3 through its N-terminal part and by binding SMC1 via its C-terminus. This ring-shape structure interacts with several additional components that regulate its functions and have the capability to encircle chromatin without a direct DNA-binding contact[100].

The canonical role of the complex is related to chromatin cohesion and chromosome segregation. It maintains the cohesion of sister chromatids from the S-phase until the onset of anaphase, to ensure an equal segregation into the two daughter cells[101].

It has also a crucial role in the DNA stability and damage response[102].

Moreover, scientific evidence has recently underlined that Cohesins directly regulate transcription of genes involved in cell proliferation, pluripotency, and differentiation. The involvement of the ring in regulation of gene expression is a more recent discovery and is defined as non-canonical role[102].

Germline mutations in the genes encoding the core cohesin subunits are implicated in several human developmental disorders, known as 'Cohesinopaties'[103, 104]. Among those, Cornelia De Lange Syndrome (CdLS) is the most common[105].

As regards somatic events, cancer genomics analyses have discovered a high frequency of mutations in Cohesins genes, as well as in genes encoding for regulatory factors, in a subset of human tumors including Acute Myeloid Leukemia (AML)[101].
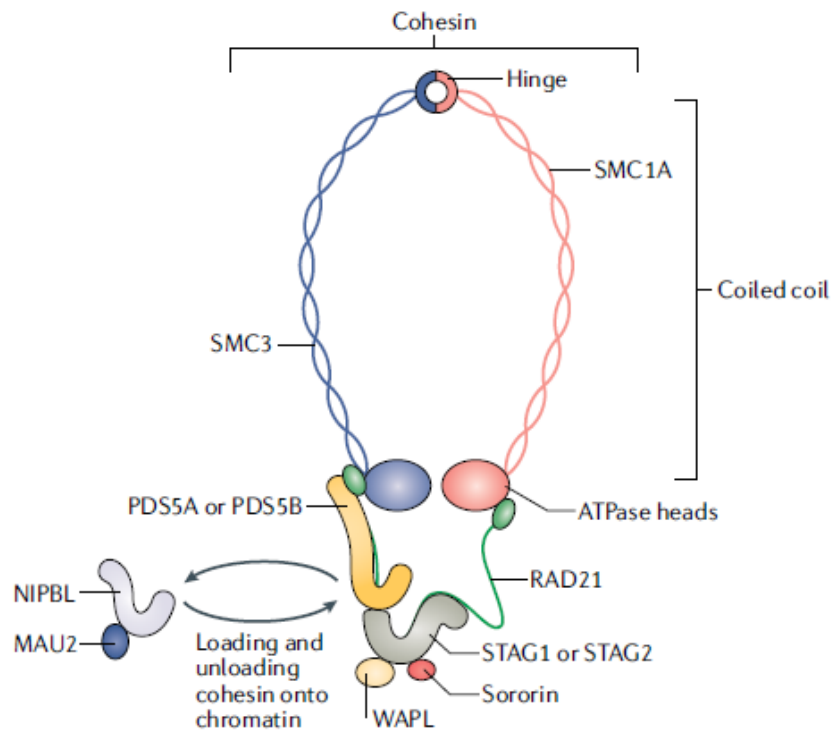

Figure 7 Schematic representation of principal proteins involved in the Cohesin complex. Adapted from Waldman, Nat Rev Cancer 2020[99]

Canonical role of the Cohesin complex

Chromatid segregation

Several cohesin regulatory factors are responsible for loading, stability, and cleavage of the complex on chromatin during cell cycle.

In detail, the cohesin ring loads onto chromatin in G1 phase of the cell cycle, immediately after cytokinesis, and remains bound specifically to centromeres in prophase. The loading is dependent on the NIPBL-MAU heterodimer (ATPase-dependent manner), and it is promoted by the WAPL, PDS5A and PDS5B proteins, that bind to chromatin-ring complex[99]. The acetylation of SMC3 by the acetyl-transferases ESCO1 and ESCO2, as well as the binding of CDCA5 (Soronin), stabilizes the strong interaction and allows the establishment of sister chromatid cohesion during DNA replication in S phase[106].

Also, the activity of STAG proteins promotes the maintenance of the structure: STAG2 subunit is essential for chromatid cohesion at centromeres and along chromosome arms, while STAG1 subunit is essential for chromatid cohesion specifically at telomeres[107].

At the onset of mitosis in early prophase, phosphorylation of STAG2 by PLK1 drives dissociation of the majority of cohesins along chromosome arms[95], while centromeric cohesion is guaranteed by binding of SGOL1 (Shugoshin). These mechanisms confer their classic X-shape to metaphase chromosomes.

Activation of the anaphase leads to degradation of PTTG1 (Securin) and to activation of ESPL1 (Separase), that cleaves the RAD21 subunit of the remaining chromatin-bound cohesion. Cohesin ring and the

centromeric cohesion are so cleaved, allowing sister chromatids to snap apart at the metaphase-to anaphase transition. Thereby chromatids can be separated into daughter cells (Fig.8)[108].
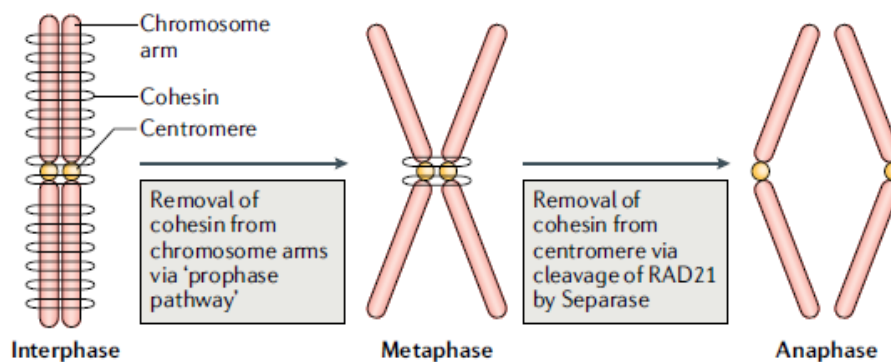
Figure 8 Localization of Cohesins during separation of sister chromatids-Canonical model of cohesin action.
Adapted from Waldman, Nat. Rev. Cancer 2020[99]

DNA Damage Repair

Among the Cohesin functions, that are crucial to preserve genome integrity, their ring guarantees the correct progression of cell cycle, and it is required for G1, intra-S and G2–M DNA Damage Checkpoints (DDC)[109].

Double strand breaks (DSB) induction leads to Cohesins accumulation near the break site, where the ring is responsible of recruitment and activation of checkpoint/DNA repair proteins. The NIPBL-MAU complex allows Cohesins enrichment around damage site, where the complex activates a network of DNA repair mechanisms to translate

checkpoint signals into DNA repair alarms, delaying the progression of cell cycle until the integrity of the double strand is re-established.

First, it promotes an efficient repair by homologous recombination (HR), providing a stable template to the sister chromatids. During S-phase, SMC subunits are phosphorylated by the damage marker ATM, thus the ring limits the synthesis of damaged DNA for the replication forks to stall. In G2/M phase, it also prevents the premature entry in mitosis under DNA damage conditions and promotes the formation of sister chromatids junctions. Chromatids are held in close proximity of stalled replication forks, in order to allow timely and efficient resumption and completion of DNA replication[109].

Finally, it suppresses damage-induced recombination and prevents joining of distal DSBs, responsible of oncogenic chromosomal aberrations (Fig.9)[110]

In support of these data, Watrin et al. demonstrated that cohesin-depleted cells are characterized by a higher number of spontaneous DNA damage events, expression of an increase in activated forms of ATM, CHK1, and H2AX, common DSB markers[111].

The complex contributes also to the structure of irradiation-induced loci.
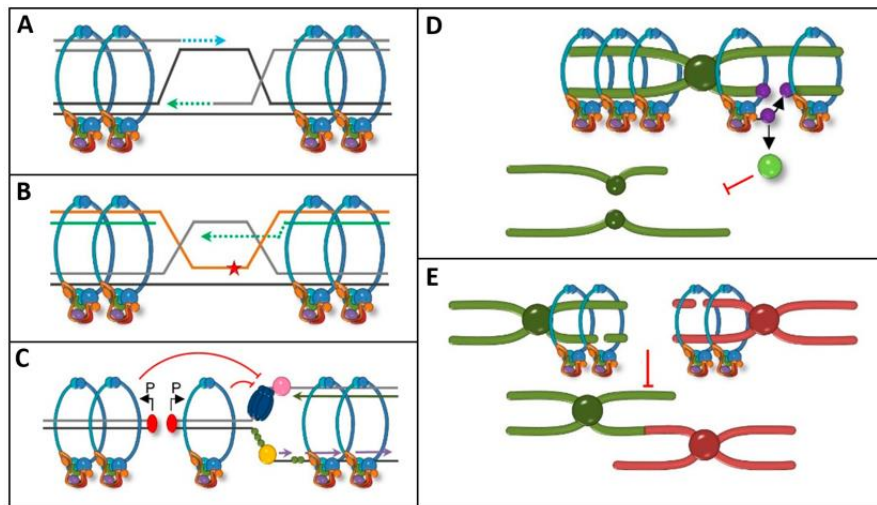
Figure 9 Cohesin functions in DNA damage response. (A) DSB repair by HR; (B) Template switch-mediated gap filing; (C) Inhibition of DNA synthesis in response to DBSs in S-phase; (D) Blocking chromatid separation through G2/M checkpoint activation; (E) Inhibition of chromosome fusion.
Adapted from Litwin, Pilarczyk et Wysocki, Genes. 2018)[109]

In summary, Cohesins' alterations may compromise chromosomes integrity, increasing the risk of genome instability, that is recognized as an oncogenesis promoting factor[110].

Non-canonical role of the Cohesin complex: regulation of gene expression

Among the several functions that Cohesin ring carries out, regulation of gene expression is certainly the most complex. Although the mechanisms are not yet fully understood, it is now known that this function is independent of the role in cohesion of sister chromatids. Thanks to their capability to contribute to chromatin architecture,

Cohesins directly regulates transcription of genes involved in cell proliferation, pluripotency and differentiation[112].

The ring interacts with the CTCF binding factor and other proteins involved in regulation of genes expression, forming, and stabilizing specific topological loops. Thus, it defines spatial conformation of specific loci, allowing long-range interactions between cohesin binding sites and defining communications between enhancers and promoters that reflect specific expression pathways[113].

Additional evidence shows that CTCF and cohesin contribute differentially to chromatin organization; CTCF brings chromosome loci closer and then Cohesins bind them together and entrap the loop in rings, stabilizing long-range interactions and facilitating transcription, as depicted in Fig. 10. Moreover, the ring is also recruited in an CTCF-independent manner, to bind target genes and promote gene transcription[112].

It has been demonstrated that Cohesins depletion is involved in loss of long-range contacts, extensive decompaction of large-scale domains, loss of intra-domain contacts and deregulation of gene expression;[114] in support of this evidence, NIPBL-mutated cells are characterized by a large number of dysregulated genes.
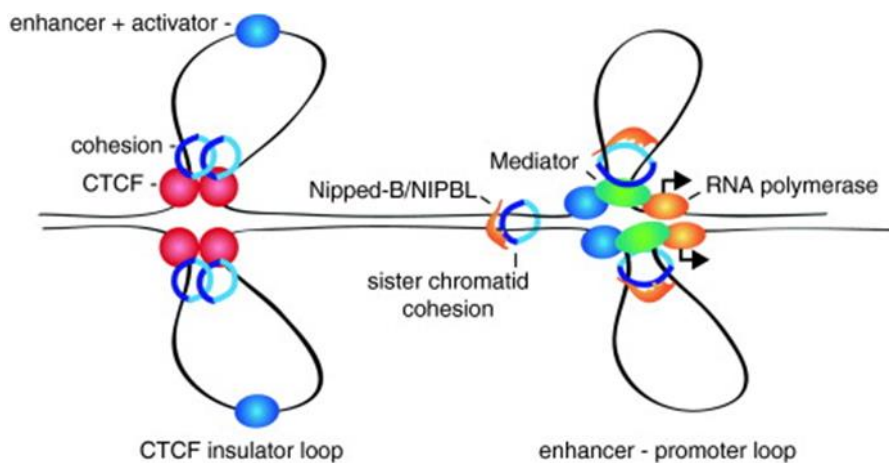
Figure 10 Cohesins capability to facilitate DNA looping.
The diagram illustrates two sister chromatids. On the left, Cohesins support intrachromosomal looping between two CTCF binding sites. On the right, the ring stabilizes a loop between an enhancer and promoter, facilitating transcriptional activation. Mediator, a transcriptional coactivator, forms a complex with Cohesin and the cohesin loading factor NIPBL loads the complex at promoters. Mediator and Cohesin occupy different promoters in different cells, thus generating cell-type specific DNA loops linked to the gene expression program of each cell[115].
Adapted from Dorsett, CurrOpin Genet Dev. 2011[102]

Cohesin genes in genetic syndromes: germline mutations

Mutations in the cohesin complex (both structural and ancillary cohesin genes) cause a multispectrum developmental abnormalities, named "cohesinopathies". This group of conditions historically included Cornelia de Lange syndrome (OMIM #122,470, # 300,590, # 610,759, # 614,701 and # 300,882), Roberts syndrome (OMIM # 268,300) and Warsaw-Breakage syndrome OMIM #613,398). However, recently, new phenotypes and clinical entities have been described: CHOPS syndrome (OMIM # 616,368) caused by mutation in

AFF4, STAG2-related X-linked Intellectual Deficiency (OMIM # 301,022) and CAID (Chronic Atrial and Intestinal Dysrhythmia) syndrome (OMIM # 616,201)[116]

Among them, Cornelia de Lange syndrome (CdLS) is the most characterized. It is an autosomal dominant disease, caused by mutation in both ancillary genes, such as *NIPBL* and *HDAC8*, and core cohesion genes, such as *SMC1A*, *SMC3*, *RAD21*. Recently also variants in *BRD4* and *ANKRD11* were described in such patients. CdLS patients share typical facial dysmorphism, microcephaly, growth delay and major malformations, such as limb reduction and heart defects. A certain degree of intellectual disability, ranging from severe to mild, is always described[117].

Biological studies, performed on CdLS patients-derived cell lines and *in vivo* models, don't demonstrated mitotic defects or premature chromatid separation, but showed a dysregulation of some of the cohesin-dependent genes[117, 118].

Cohesin genes in hematological malignancies

Somatic mutations in Myelodysplastic syndromes (MDS) and Acute Myeloid Leukemia (AML)

Cohesin genes are frequently affected by somatic events in cancer[119]. Alterations in the genes encoding the core cohesin subunits or its regulatory factors have been reported in several tumors, included myelodysplastic syndrome (MDS) and Acute Myeloid

Leukemia (AML). They occur with high frequency in patients with myeloid neoplasms (12% of cases) (Fig.11), where they are often mutually exclusive and lead to decreased function of the Cohesin complex[120].

Cohesin defects are most prevalent in high-risk MDS and secondary AMLs and are associated with poor overall survival, especially in *STAG2* mutant MDS patients[120].

Those somatic variants are nonsense and frameshift aberrations that occur early in disease development, in expanding subclones, and co-occur with other mutations known to be drivers of clonal evolution. Analysis for clonal hierarchy performed by Thota et al.[118] demonstrated that mutations in *STAG2*, *SMC3*, and *RAD21* are often ancestral, and they expand to clonal dominance concordant with disease progression. They are often responsible of dysfunction of the checkpoint proteins (as MAD2 and/or BUBR1), with consequent chromosome segregation and DNA repair transaction defects, exacerbating the genomic instability commonly associated with different type of cancers[120].

Cohesin mutations are early, but not initiating, genetic lesions during myeloid disease development. They give a clonal advantage and facilitate a positive mutational selection, predetermining the types of additional secondary mutations that result in evident leukemic transformation.[120]

Somatic mutations of the Cohesins alone are often insufficient to impart complete malignant transformation. They act in epigenetic regulation with co-occurring mutations in chromatin modifiers (as

ASXL1); however, they have effect on gene expression dysregulation if simultaneously present with aberrations in key transcriptional regulators (*RUNX1*, Ras family genes, and *BCOR*)[121].
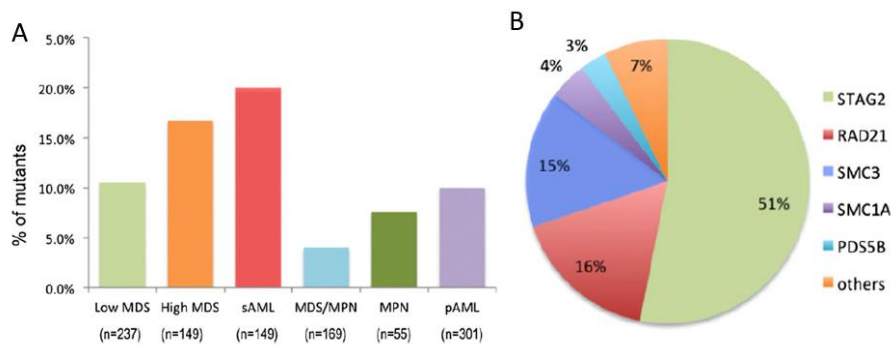
Figure 11 Characterization of Cohesin mutations in patients with a myeloid disease. (A) Frequency of Cohesin mutations in each myeloid malignancy in Thotas' cohort: 10.5% of lower-risk MDS patients, 16.8% of High risk MDS patients, 20.1% of secondary AML, 4.1 % of MDS/MPN, 7.3% of MPN, 10.6% of primary AML. (B) Distribution of Cohesin mutations identified across the patient cohort.
Adapted from Thota et al, Blood, 2014[122]

Therefore, cohesin defects resulted in alteration in chromatin architecture and deregulated expression of genes involved in myeloid development and differentiation, with enhanced effects on self-renewal of hematopoietic stem and progenitor cells. It has been recently demonstrated that depletion of Cohesins severely impairs the expression of *ETV6*, a key transcription factor in self-renewal programs: the failure activation of its repressor Ets abrogates induction of erythroid transcriptional programs of differentiation.[83,123]

So far, somatic Cohesin mutations have not been associated with aneuploidy or complex cytogenetics[122].

Due to this line of evidence, targeting of cohesin complex is a promising area of drug development, still underexplored[123].

Potential role of germline mutations in predisposition to hematological disease

To date, a direct cause-effect correlation between genetic syndromes and oncogenesis is not established yet; in addition, no evidence is sufficiently significant to define Cohesinopaties cancer-prone syndromes.

Despite this fact, in the last decades, multiple case reports supported a role of Cohesins' germline mutations in predisposition to neoplasms development.

A 23-month-old child affected by Roberts syndrome that developed a sarcoma botryoides,[124] in addition with a case of melanoma in a girl with Roberts-SC Phocomelia Syndrome[125], suggested a possible correlation between Cohesinopaties and increased risk of malignancy. In the hematological field, Vial et al.[126] hypothesized for the first time that germline mutations in Cohesins could constitute a predisposing factor to leukemia. They described a single case of Down syndrome-like Acute Megakaryoblastic Leukemia (AMKL) in a patient with Cornelia de Lange syndrome (CdLS), in which a pre-leukemic clone combines a constitutional *NIPBL* mutation with somatically acquired trisomy 21 and *GATA1* mutation.

Moreover, our group recently described the first CdLS patient with ALL,[127] carrying a *NIBPL* mutation, as described in State of the art 1 paragraph. The analysis of the family indicated a *de novo* origin of this novel deleterious variant[127].

In support of the connection between Cohesinopaties and Leukemia, in a large cohort of children with CdLS cancer accounted for 2% of deaths, highlighting a slightly increased of cancer risk compared to the healthy population[128].

Genetic mechanisms through which germline Cohesin mutations could perturb hematopoietic development are not clear yet. Considering the role of the complex in gene expression and DNA repair, loss of Cohesins functions might lead to genetic instability in progenitor cells, that become more susceptible to DNA damage and leukemic transformation. The underlying processes still need to be established and further explored.

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2. Scope of the thesis

The aim of this work was to investigate the role of predisposition in pediatric acute lymphoblastic leukemia, which according to recent findings occurs in about 5% of cases[1] [2-5].

In this work, we aimed both to extend the phenotype of known disease and to uncover new possible associations between cancer predisposition and ALL.

The work was developed in 3 different tasks:

1. Incidence and therapeutic implications of germline TP53 mutations in hypodiploid childhood acute lymphoblastic leukemia: a retrospective analysis of the Italian cohort

We describe a large cohort of low hypodiploid ALL patients tested for TP53 variants. We confirm a high frequency of deleterious TP53 mutations that correlate with an increased risk of family history cancer and second malignancies. We conclude that TP53 mutation testing is warranted in patients with hypodiploid ALL to ensure appropriate genetic counseling for patients with germline mutations and their families and tailored clinical surveillance (according to the Li-Fraumeni syndrome guidelines).

2. First description of a *PAX5* germline variant with frameshift in two siblings with B-cell precursor acute lymphoblastic leukemia

We report a potentially diverse mechanism contributing to leukemogenesis associated with *PAX5* germline mutations by describing the first germline frameshift *PAX5* variant in a family with recurrence of BCP-ALL.

3. Potential Role of *STAG1* Mutations in Genetic Predisposition to Childhood hematological malignancies.

We investigated the effects of two rare *STAG1* germline variants (Arg1167Gln and Arg1187Gln) in two pediatric patients affected by Acute Lymphoblastic Leukemia (ALL) and Myelodysplastic syndrome (MDS), respectively. We characterized their position along the gene sequence and protein domains considering conservation and mutational landscape. In addition, we investigated the functional consequences of the *STAG1* mutation in a lymphoblastoid cell line (LCL), a preclinical in vitro cell model that allows assessment of chromosome stability and DNA repair mechanisms. These mechanisms typically represent impaired processes in oncogenesis and are therefore essential for assessing how the variant might contribute to tumor transformation.

The common goal of all tasks was to improve the understanding of oncogenesis and to open new scenarios regarding the contribution of genetic predisposition to hematologic malignancies.

Overall, better knowledge and characterization of predisposing genetic alterations could enable targeted surveillance strategies that impact both genetic family counseling and direct patient care, including treatment conditions and hematopoietic stem cell transplantation (HSCT)

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Chapter 3

(Manuscript in preparation)

Incidence and therapeutic implications of germline *TP53* mutations in hypodiploid childhood acute lymphoblastic leukemia: a retrospective analysis of the Italian cohort

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1.   Abstract

Childhood hypodiploid Acute Lymphoblastic Leukemia (ALL) represents a rare and challenging subtype of ALL with a poor prognosis despite intensive therapies. *TP53* germline variants are known to be associated with Li-Fraumeni syndrome (LFS). This study delves into the Italian cohort of hypodiploid ALL patients, examining the presence of germline *TP53* mutations and their clinical implications. Using a custom predisposition NGS panel, *TP53* variants were identified in 52% of pediatric hypodiploid ALL cases. Notably, 63% of these TP53 variants were germline. Patients with *TP53* variants were diagnosed at older ages, primarily in the low hypodiploid subgroup. Second malignancies occurred significantly more often in patients with germline *TP53* mutations, especially after hematopoietic stem cell transplantation (HSCT). Furthermore, germline *TP53* mutations were associated with a higher incidence of cancer in young family members.

In conclusion, this study contributes valuable insights into the genetic landscape of childhood hypodiploid ALL and emphasizes the importance of considering germline *TP53* mutations in treatment decisions and long-term care.

3. Introduction

Childhood hypodiploid Acute Lymphoblastic Leukemia (ALL) is a rare subtype of ALL characterized by a poor prognosis despite intensive chemotherapy treatment and hematopoietic stem cell transplantation (HSCT)[1-3]. It may be further categorized in high hypodiploid, low hypodiploid and near-haploid based on karyotype or DNA index (DI)[4, 5]. Previous studies have shown that low-hypodiploid ALL show a high frequency of TP53 pathogenic variants that were proved to be germline in approximately half of the cases[1, 6]. *TP53* germline variants cause Li-Fraumeni syndrome (LFS, OMIM #151623) that is a well-known cancer predisposition syndrome associated with an increased risk for childhood- and adult-onset malignancies; the lifetime risk of developing cancer in individuals affected with LFS is ≥70% for men and ≥90% for women[7-9]. The five most common cancers that occur in this condition are adrenocortical carcinomas, breast cancer, central nervous system tumors, osteosarcomas, and soft-tissue sarcomas[7]. Moreover, LFS is associated with an increased risk of several other cancers including hematological malignancies[7, 10].

In this study we retrospectively dissect germline *TP53* mutations in hypodiploid ALL in an Italian cohort, considering patients enrolled in the last three front-line protocols, focusing and investigating clinical features of LFS, such as second malignancies and family history of cancer.

Material and methods

Samples selections

The study involved retrospective selection of pediatric hypodiploid patients (< 18 years of age) enrolled in four nationwide frontline protocols: ALL AIEOP-BFM 2000, ALL AIEOP-BFM 2009, ALL AIEOP-BFM 2017 observational, ALL AIEOP-BFM 2017. For all patients, demographic data, cytogenetics records, disease characteristics, and data about toxicity were prospectively collected.

For all selected patients we performed the NGS analysis in disease-hematopoietic tissue; subsequently, we performed the same analysis in germline tissues (DNA extracted from bone marrow at remission defined as negativity by PCR-MRD, 10-4 sensitivity[11] or buccal brush samples) of selected patients, on the basis on the results obtained on the diseased tissue.

Ethic statement

Samples were obtained from patients, after a written informed consent from parents or legal representatives. The study was approved by each institutional review board and conducted in accordance with the ethical standards of the Declaration of Helsinki and to national and international guidelines.

Custom predisposition NGS panel

NGS analysis was carried out with a custom gene panel, consisting of 39 genes that have been mainly associated with leukemia predisposition (Supplementary Table 1).

The custom panel was designed with the Integrate DNA Technology (IDT) platform (xGen Predesigned Gene Capture Pools – https://idtdna.com/site/order/ngs), generating high fidelity single strand DNA probes. It consists of 1520 probes and a cumulative targeted region of 141 kb.

Target-Capture DNA Next Generation Sequencing Target sequencing was performed on genomic DNA using Nextera Flex for Enrichment by Illumina protocol (#1000000048041 v01). The size range and quality of the library were measured both with 2100 Bioanalyzer Systems (Agilent Technologies) - DNA 1000 Kit and Qubit™ dsDNA Quantification Assay Kits, high sensitivity.

The pool libraries were paired end (2x150) sequenced on flow cell with v2.5 chemistry on Nextseq550 (Illumina) instrument. FASTQ files were generated by Local Run Manager software.

Data Analysis

The sequencing process was subjected to matrix monitoring where quality statistics such as data intensity, cluster density, Qscores were tabulated within the BaseSpace platform. After sequencing, a demultiplex was performed to generate FASTQ files for each sample from bcl2fastq format file, ready for computational analysis. The FASTQ files were then analyzed using the Sophia DDM software. Alignment was performed against the Human Reference sequence

GRCh37/Hg19. Variants were filtered by variant fraction (VF) >5% and coverage at least 500X; Variant Allele Fraction (VAF) in the population was set at 1%. We included certainly pathogenic, potentially pathogenic and variants of unknown significance (VUS). Novel exonic non-synonymous variants were also retained. Benign/likely benign variants in all databases of prediction are excluded from the results. The most common databases of prediction were consulted for the interpretation of the pathogenicity, including ClinVar, Clinical Genome, IARC *TP53* database[12], COSMIC, Varsome, InterVar.

Copy Number Variation (CNV) analysis was not performed.

Statistical analysis

P values were determined using the Fishers exact test; STATA analysis software was used to plot and perform statistical analysis.

Kaplan Meier curves were used to determine Event Free Survival (EFS) and Cumulative Incidence (CI). The p values were determined through the log rank test. In both cases differences were considered statistically different if p values < 0,05.

Results

We selected 42 cases of pediatric hypodiploid BCP-ALL patients recruited into four nationwide frontline AIEOP protocols from December 2000 to January 2020. Among the 42 elected patients, 16 were enrolled in the ALL 2000 protocol, 18 in the ALL 2009, 4 in the ALL 2017 observational and 4 in the ALL 2017 (Table 1).

ID	Sex	Age	AIEOP Protocol	IP	DNA index	Karyotype	HSCT	Relapse
01	M	15	ALL_2000	B-II	0,584			
02	M	4	ALL_2000	B-II	0,798			
03	M	5	ALL_2000	B-II	0,796			
04	F	7	ALL_2000	B-II	0,517			
05	F	14	ALL_2000	B-III	0,788			
06	M	6	ALL_2000	B-I	0,59		Y	VE
07	F	2	ALL_2000	B-I	0,77			
08	M	11	ALL_2000	B-II	0,79		Y	
09	F	8	ALL_2000	B-III	0,52			
10	M	6	ALL_2000	B-II	0,52			VE
11	M	9	ALL_2000	B-II	0,71			VE
12	F	6	ALL_2000	B-II	0,59			VE
13	M	14	ALL_2000	B-III	0,75			
14	M	9	ALL_2000	B-II	0,76		Y	VE
15	F	9	ALL_2000	B-II	0,79			
16	F	2	ALL_2000	B-II	0,78			
17	M	14	ALL_2009	B-II	0,7			
18	M	15	ALL_2009	B-II	0,7	33,X,-Y,-2,-3,-4,-7,-9,-12,-13,-14,-15,-16,-17,-20[14]/46,XY[2]		VE
19	F	6	ALL_2009	B-II	0,78	68~72,XX,+X,+X,+1,+1,+5,+5,+6,+6,+8,+8,+10,+11,+11,+12,+12,+14,+18,+18,+19,+ 19,+20,+20,+21,+21,+22,+22[cp14]/46,XX[6]		
20	M	3	ALL_2009	B-II	0,58		Y	
21	F	14	ALL_2009	B-II	0,57			L
22	M	1	ALL_2009	B-II	0,76	46,XY,i(9)(q10),der(19)t(1;19)(q23;p13)[14]/46,XY[6]		
23	F	12	ALL_2009	B-II	0,71	32~33,X,-X,-2,-3,-4,-7,-12,-13,-14,-15,-16,inc[cp6]/46,XX[27]		
24	M	12	ALL_2009	B-II	0,77	46,XY[11]/66,XXY,+X,+Y,+1,-2,-3,-4,-9,-10,+11,-12,-13,-17,+18,+21,+22[12]/68,XXY,+Y,+1,-2,-3,-5,-9,-13,-14,-17,+19,+21+22[7]	Y	
25	F	2	ALL_2009	B-II	0,77			
26	F	9	ALL_2009	B-II	0,78	72<3n>,XXX,+1,-2,-3,+6,+18,+21,+22[3]/46,XX[15]	Y	VE
27	F	3	ALL_2009	B-II	0,56			L
28	F	12	ALL_2009	B-II	0,78			
29	F	13	ALL_2009	B-II	0,53		Y	VE
30	F	6	ALL_2009	B-II	0,56		Y	VE
31	F	1	ALL_2009	B-III	0,59	20~27[7]/52~55,XX[5]/46,XX[18]		L
32	M	12	ALL_2009	B-III	0,71	46,XY		
33	F	13	ALL_2009	B-II	0,7		Y	
34	M	5	ALL_2009	B-III	n.a.	35,XY,-2,-3,-4,-7,-9,-12,-13,-15,-16,-17,-20[3]/68,idemx2,-5,-10,-14,+mar[1] /46,XY[19]	Y	
33	M	12	ALL_2017obs	B-III	0,52			
34	F	9	ALL_2017obs	B-III	0,76		Y	VE
35	F	13	ALL_2017obs	B-II	0,74	34,X,-X,-2,-3,-4,-7,-12,-13,-14,-15,-16,-17,-20[18]/46,XX[10]	Y	
36	F	15	ALL_2017obs	B-II	0,76			
37	F	15	ALL_2017	B-II	0,76	32-39XX, -2,-4,-12,-13-17,-19,inc[cp8]/63-67,xx,+1,+1,+21,+21,inc[cp5]/46,xx[22]	Y	
38	M	15	ALL_2017	B-II	n.a	34,XY,-2,-3,-4,-6,-7,?del(8)(q22),-10,?del(12)(p13),-12,-13,-14,-15,-16,-17,-22,+mar[11]/46,XY[9]	Y	

Table 1

Analysis of TP53 variants

All 42 patients were screened for *TP53* variants by performing a targeted Next Generation Sequencing (NGS) Nextera Flex DNA panel of 39 ALL predisposition genes. *TP53* NM_001276696.1 was used as reference sequence.

TP53 variants were observed in 22/42 (52%) patients. Overall, we filtered 20 variants, since three patients shared the same variant. 14/20 were SNP, while 6/20 were indel; 13/20 were missense, 3/20 were frameshift, 2/20 were nonsense, 2/20 were inframe.

14/20 variants were classified as pathogenic according to international databases and ACMG, 6/20, instead, were classified as variant of unknown significance (VUS). Considering the VUS, 3/6 were missense, 2/6 in-frame 1/6 frameshift.

As expected, most of the variants fall in the DBD (16/20), while 3/20 in the OD domain and 1/20 in the nuclear localization signal (NLS) domain. Among the VUS, 5/6 reside in the p53 core DBD.

TP53 variants were observed mostly in low-hypodiploid patients (DNA index ranging from 0,7-0,78); only one patient with a *TP53* variant presented a DI of 0,58. Patients with no *TP53* variants, instead, were near-haploid (DNA index 0,51-0,59) and low hypodiploid in 6/20.

14/22 (63%) were confirmed to be germline variants, 8/22 (25%) were somatic. Among the germline variants, 1 was found to be a germline mosaicism, presenting a *TP53* variant both in remission (VF 10%) and buccal brush sample (VF 8,5%).

Among the somatic variants, 5/8 were classified as pathogenic, 3/8 as VUS. 12/14 germline variants were pathogenic, while 2/14 were considered VUS.

Comparing our variants with what reported in the IARC (International Agency for Research on Cancer) *TP53* database and the COSMIC database, 3 somatic variants (c.799_800insCTG, c.844_845insAAAT, c.811_812insCCAGTAAGCC) were not recorded.
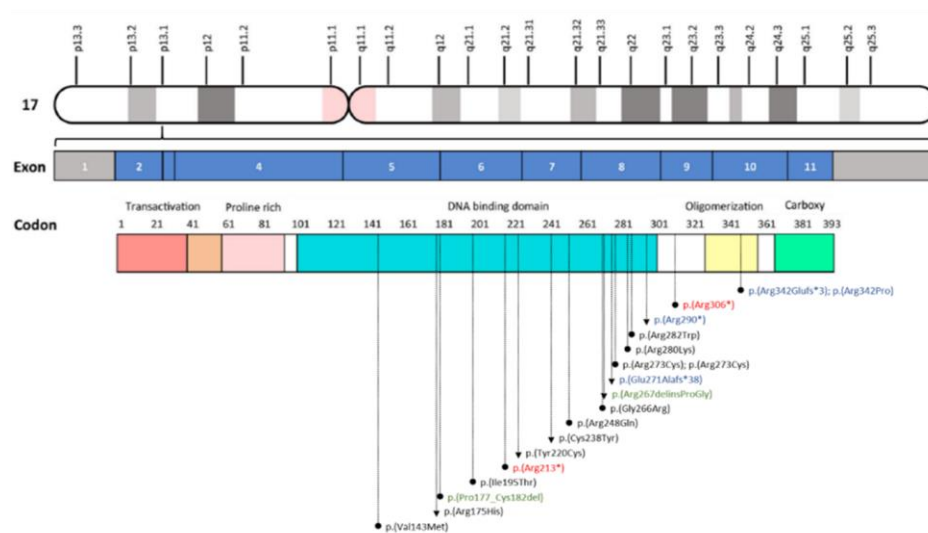
TP53 variants are summarized in Fig. 1 and Table II.

Figure 1: *TP53 gene organization and distribution of mutations by codon and domain. Most of the variants cluster within the DNA-binding domain (codons 100–300, exons 4–8. Black: missense variants; Red: nonsense variants; Blue: frameshift variants, Green: inframe variants. ●:germline variants▲:somatic variants. Adapted from Campo et al., 2018.*

ID	TP53 score	Origin	Type	Coding conseguence	c.DNA	Protein
05	PV	Somatic	SNP	missense	c.524G>A	p.(Arg175His)
13	VUS	germline	INDEL	missense	c.795_796delinsAA	p.(Gly266Arg)
14	VUS	Somatic	INDEL	frameshift	c.866_867insGTGACCCGAATCT	p.(Arg290*)
15	PV	germline	SNP	nonsense	c.916C>T	p.(Arg306*)
16	VUS	germline	SNP	missense	c.427G>A	p.(Val143Met)
17	VUS	germline	SNP	missense	c.839G>A	p.(Arg280Lys)
18	PV	germline	SNP	missense	c.529_546del	p.(Pro177_Cys182del)
19	PV	germline	SNP	missense	c.817C>T	p.(Arg273Cys)
23	PV	Somatic	SNP	missense	c.659A>G	p.(Tyr220Cys)
24	PV	germline	SNP	missense	c.743G>A	p.(Arg248Gln)
25	PV	germline	INDEL	frameshift	c.1024del	p.(Arg342Glufs*3)
26	PV	germline	SNP	missense	c.844C>T	p.(Arg282Trp)
28	PV	somatic	SNP	missense	c.713G>A	p.(Cys238Tyr)
30	VUS	somatic	INDEL	inframe_3	c.799_800insCTG	p.(Arg267delinsProGly)
32	PV	germline	SNP	missense	c.817C>T	p.(Arg273Cys)
33	PV	germline	SNP	missense	c.817C>T	p.(Arg273Cys)
34	PV	germline	SNP	missense	c.1025G>C	p.(Arg342Pro)
36	PV	somatic	SNP	missense	c.818G>T	p.(Arg273Leu)
37	PV	germline	SNP	missense	c.584T>C	p.(Ile195Thr)
38	PV	somatic	INDEL	frameshift	c.811_812insCCAGTAAGCC	p.(Glu271Alafs*38)
39	PV	germline	SNP	nonsense	c.637C>T	p.(Arg213*)
40	VUS	germline	INDEL	inframe_18	c.529_546del	p.(Pro177_Cys182del)

Table II: List of *TP53's variant identified in the cohort of hypodiploid ALL patients. TP53 variants were classified according to TP53 Variant Curation Expert Panel (VCEP) indications*

Abbreviations: PV pathogenic variant; VUS variant of uncertain significance; SNP single nucleotide polymorphism.

Clinical features

Among the selected cohort, median age at time of diagnosis was 9 years (range 1-17); 21 patients (52%) were female while 19 patients (48%) were male. Patients with a *TP53* variant were diagnosed with ALL at an older age, compared to hypodiploid patients without *TP53* variants (median age at diagnosis respectively 11 years compared to 7 years, p. value: <0,05). No differences between genders were observed.

Patients were stratified and treated according to the stratification criteria of the different protocols: patients enrolled in ALL 2009, 2017 observational and ALL 2017 were all considered high risk (HR); among patients enrolled in the ALL 2000, instead, 5/16 were classified as standard risk (SR), 10/16 medium risk (MR), 1/16 HR.

Sixteen out of 42 patients underwent a bone marrow transplant (HSCT) in first clinical remission. Overall, 13/42 patients relapsed: 10 were very early relapses (< 18 months from diagnosis), and 3 were late relapses (> 30 months from remission). Relapses were reported more frequently in the wild-type TP53 group and mainly in patients with a near haploid DI. Five second malignancies (SMN) were reported (1 acute myeloid leukemia, 3 osteosarcoma, 1 liposarcoma); interestingly, all second malignancies occurred in patients with a germline tp53 pathogenic variant and all in patients who underwent a HSCT (p <0,001, evaluated with Fischer test). The latency between ALL diagnosis and SMN onset was of 5,8 at median (range 3-9 years from first diagnosis). Patients' clinical features are summarized in table 1.

Focusing on the family history of our cohort, we highlighted that the germline *TP53* mutated cohort showed a higher incidence of cancer in young age (< 45 years of age at diagnosis) (Fig. 2).
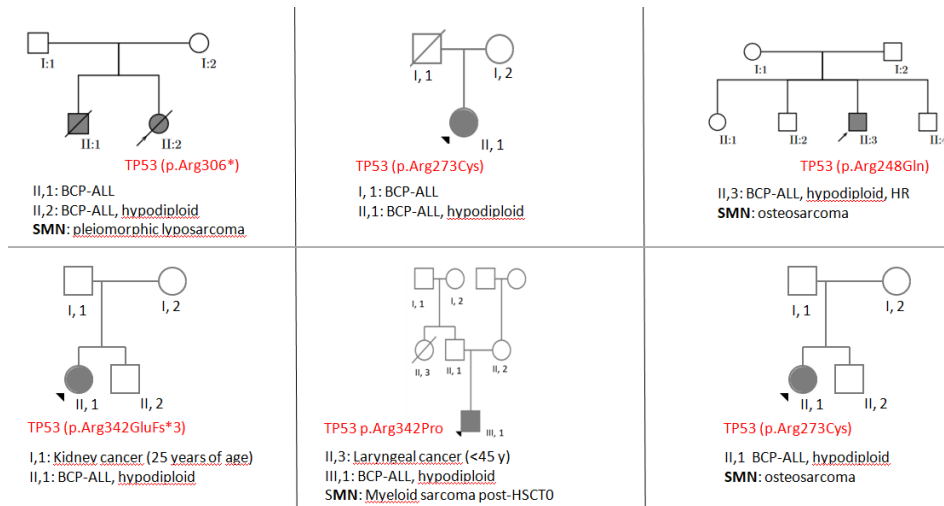

Figure 2: Examples of family-trees of patients with germline TP53 mutation showing an increased frequency of secondary cancers and family history of cancer.

EFS and OS were evaluated considering the wild-type TP53 patients together with patients carrying VUS versus patients carrying a pathogenic TP53 variant. Considering the whole cohort the prognosis was dismal. No differences were observed in terms of EFS between the two groups (TP53 wild-type 49,1% vs TP53 mutated 46,4%) (Fig. 3A) and OS (TP53 wild-type 60% vs P53 mutated 60,6%) (Fig. 3B).

3A

3B
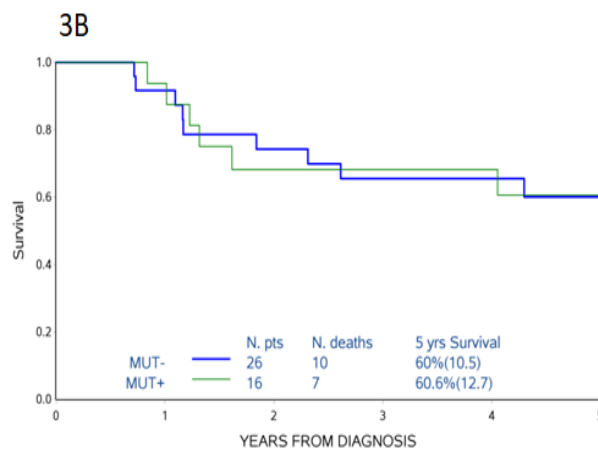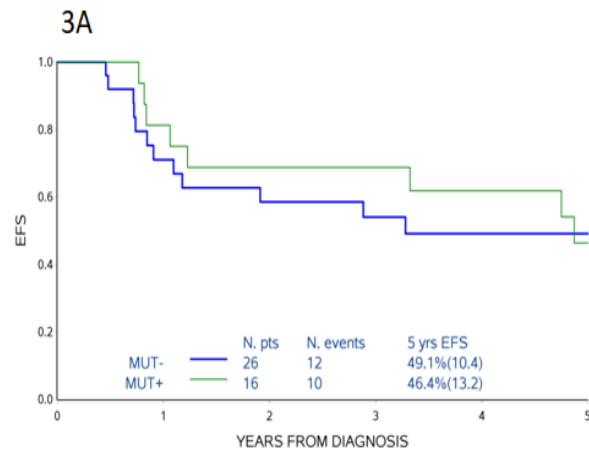
Figure 3: EFS and OS in TP53 wild type cohort (mut -; blue) vs TP53 mutated cohort (mut+; green). Patients with VUS were included in the wild-type cohort. No statistically significant difference was observed between the two groups.

Discussion

Our data represent the largest hypodiploid patient cohort tested so far. As expected, we found two distinct mutational profiles between low hypodiploid and near haploid ALL patients. Our data show that *TP53* is

the most frequent mutated gene, as it is found altered in 50% of the screened cohort, predominantly in low-hypodiploid patients. Basing on our previous data (data not shown), the frequency of *TP53* variants in hypodiploid ALL is found to be significantly higher compared to the one observed in mutational analysis of non-hypodiploid ALL: in our institution we sequenced 200 consecutive diagnosis of ALL and TP53 variants were detected in less than 1% of the cases (data not shown). According to previously published studies describing TP53 somatic or germline mutation in other cancers[13], the *TP53* variants identified in our study show a restrictive pattern of distribution as all but two reside within the DNA-binding domain, confirming that an aberration of the DBD is crucial during leukemogenesis. The strict relationship between TP53 variant and hypodiploidy prompts the hypothesis that a loss of function in p53 triggers DNA instability and the hypodiploid leukemia phenotype.

Interestingly, 65% of *TP53* variants were found to be germline, consistent with what reported by Comoeaux and Mullighan [6] and Qian and colleagues [14]. Therefore, our data reinforce the evidence that childhood hypodiploid ALL is one of the possible manifestations of Li-Fraumeni syndrome (LFS). In contrast, germline *TP53* mutations in adult ALL are extremely rare[15], suggesting that the relation between LFS and lymphoblastic leukemia may be predominantly confined to childhood.

In our cohort of patients, the presence of a *TP53* variant did not confer a worse prognosis compared to hypodiploid the wild-type TP53 group, both in terms of 5 years EFS and cumulative incidence. Although a larger

cohort may need to be analyzed, our results suggest that hypodiploidy itself, rather than the specific genetic mutation, may be the strongest poor prognosis factor. The negative impact of *TP53* variants on ALL prognosis is exceeded by the concomitant hypodiploidy, which is known to correlate with an elevated risk of relapse and/or failure of therapy[3, 14, 16, 17]. However, our results clearly show that patients carrying a TP53 germline variant have a significantly increased risk of developing second malignancies that are known to have a dismal prognosis.

The impact of the identification of a germline *TP53* mutation in a patient affected with hypodiploid acute lymphoblastic leukemia is important at several levels. First, the evidence of a germline *TP53* mutation imposes genetic counseling for the patient and family members[18].

Secondary, hypodiploid ALL patients are considered high risk in the current protocol due to their poor outcome and, thus, treated with intensive chemotherapy and frequently eligible to HSCT. In this context, the presence of a germline *TP53* variant could expose the patient to a significant added risk for the development of treatment secondary malignancies (t-SMN), in addition to the genotoxic treatment received during ALL treatment and condition regimens (Total body irradiation)[19, 20]. Our data reinforce the evidence published by Qian and colleagues where most patients with a germline loss of function TP53 that experienced a SMN had received HSCT[14]. Certainly, to achieve better treatment guidelines it would be crucial to include germline loss of function TP53 patients in prospective clinical trials, allowing for the collection of extensive clinical data.

Third, the knowledge of a familiar pathogenic *TP53* variant must be considered during HSCT donor selection, to avoid familiar members that could eventually carry the variant[21]. Finally, patients with a pathogenic germline *TP53* variant are exposed to a high risk of second tumors, regardless of treatment-related malignancies. For this reason, these patients should be referred and centralized to specialized institutions able to judge the opportunity of a specific follow-up[22-24].

The best therapeutic regimen for hypodiploid patients is still a matter of debate, with MRD at the end of induction and TP53 germline mutations potentially indicating a highest relevance in the indication to HSCT[25]. Chemotherapy-only treatment could be indicated for MRD-low patients and HSCT for MRD-High, potentially considering a reduced conditioning regimen for TP53 mutated cases.

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Supplementary

Supplementary Table 1

Tumor suppressor and DNA repair	Transcription	RAS pathway signaling	Other signaling	Cohesins	Chromatin binding and remodeling
ATM	AUTS2	BRAF	ANKRD26	BRD2	ARID5B
BLM	CEBPA	KRAS	JAK2	BRD4	CREBBP
CDKN2A	CEBPE	NF1	PIP4K2A	HDAC8	EP300
CDKN2B	ETV6	NRAS	SH2B3	NIPBL	EZH2
NBN	GATA1	PTPN11		RAD21	NSD1
TP53	GATA2			SMC1A	PHF6
	GATA3			SMC3	
	PAX5			STAG1	
	RUNX1			STAG2	

Supplementary Table 1: List of genes included in the custom NGS panel. Classification of genes included in custom Next Generation Sequencing panel in different classes according to their biological functions.

Chapter 4

Description of a novel frameshift *PAX5* germline variant in two siblings with B-cell Precursor Acute Lymphoblastic Leukemia

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Abstract

The *PAX5* gene plays a crucial role in B-cell development and is frequently altered in B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Recently, missense *PAX5* germline variants have been described in families with recurrence of BCP-ALL. Here, we report a novel germline PAX5 frameshift variant, in a family with recurrent BCP-ALL. Surprisingly, and differently to what previously described, both patients shared the same PAX5 P80R somatic variant as a second hit.

This report sheds light on potentially diverse mechanisms contributing to leukemogenesis in the context of *PAX5* germline mutation. Thus, these findings significantly contribute to the understanding of ALL pathogenesis and have implications for familial genetic counseling.

Introduction

The *PAX5* gene, which encodes a B cell-cell related transcription factor, is altered in approximately 30% of cases of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) [1-3]. Recently, PAX5 somatic alterations (PAX5alt) have been described in both childhood and adult cohorts, as a heterogeneous group that includes cases carrying either *PAX5* fusion genes or the missense PAX5 variants, with P80R representing the most prevalent. Overall, PAX5alt and PAX5 P80R account for 7% of B-other ALL and play an important role in leukemogenesis, prognosis, and risk stratification [3-5].

Recently, *PAX5* has been identified as one of the genes that confer high penetrance susceptibility to childhood BCP-ALL [6-8] and germline missense variants have been described in families with recurrence of ALL [9-14]. Currently, 4 different *PAX5* germline variants (p.Gly183Ser; p.Gly183Arg; p.Arg38His; c.1013-2A>G, PAX5 exone 6 deletion) have been described [9-15] in 26 patients belonging to 13 different families, with incomplete penetrance of the phenotype. All family members who developed BCP-ALL had a second somatic hit affecting the wild-type PAX5 allele, either through somatic uniparental disomy following chromosome 9p deletion or as a second independent variant [9-15].

Here we report on a family with a new combination of *PAX5* germline and somatic variants. For the first time, we describe a novel frameshift *PAX5* germline variant in two Italian siblings with BCP- ALL, suggesting a possible different mechanism for promoting leukemogenesis.

Material and Methods

Patients' description

Case A was a 13-year-old boy diagnosed with BCP- ALL, without central nervous system (CNS) involvement and negative for recurrent fusion genes. He was enrolled in the AIEOP-BFM ALL2009 treatment protocol and was classified as an intermediate-risk patient based on minimal residual disease (MRD). Eight years later, his sister (14 years, designated case B) was diagnosed with BCP- ALL, was CNS-negative, negative for recurrent fusion genes, and was enrolled in the intermediate-risk group of the AIEOP-BFM ALL2017 protocol. Both patients were otherwise healthy and descended from healthy, unrelated parents; there was no history of cancer nor significant hematologic disorders in the pedigree.

Samples were collected from the patients (bone marrow, BM) and their relatives (peripheral blood, PB) after written informed consent was obtained. The study was approved by the institutional review board and was conducted in accordance with the ethical standards of the Declaration of Helsinki and with National and International guidelines.

NGS analysis

First, we screened the two siblings by a custom Next Generation Sequencing ALL predisposition panel of 99 genes, sequencing DNA obtained from BM at both disease onset and remission phase (defined as negativity by PCR-MRD, 10-4 sensitivity). The custom NGS panel was

developed using the IDT platform (xGen Predesigned Gene Capture Pools – https://idtdna.com/site/order/ngs), which generates high-purity single-stranded DNA probes.

Whole-exome sequencing was then performed on the germline DNA of the patients and both parents, exploiting Integrated DNA Technology (IDT) xGen Exome Research Panel v1.0 probes.

Libraries (both for the custom panel and WES) were prepared according to the Nextera Flex for Enrichment protocol and sequenced on the Illumina Nextseq550 platform (Illumina) in 2x150 paired-end. FASTQ files were prepared using Local Run Manager software and are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress).

Single nucleotide variants (SNVs) and insertions/deletions (indels) were identified using Congenica v.3.0 software; alignment was performed with the human reference sequence GRCh37/Hg19.

Sanger sequencing

The variants of interest were first validated and then examined in the family tree by PCR and Sanger sequencing. Primers are listed in Supplementary Table I.

Longs Reads Sequencing

To evaluate whether the identified germline and somatic variants were in *cis* or in *trans,* we performed a long read sequencing using Nanopore technology. First, we obtained cDNA from samples at onset. Through RT-PCR a cDNA fragment of 604 bp, including both variants,

was obtained. Primers are listed in Supplementary Table I. The amplicon was purified with 1x AMPure XP Beads (Beckman Coulter) and subjected to end-repair by NEBNext FFPE DNA Repair Kit (New England Biolabs) according to the standard protocol. After a second round of purification with 0.6x AMPureXP Beads, adapters were ligated using the Genomic DNA Ligation kit (Oxford Nanopore Technologies). Then the library underwent a final round of purification with AMPure XP Beads and 50fmol were loaded in a R9.4.1. flowcell according to manufacturer instructions and sequenced using a MinION MK1B sequencer (Oxford Nanopore Technologies).

Raw Nanopore fastq reads were aligned to the human GRCh38/hg38 reference using Minimap v2.24. Sam files were converted to the bam format, sorted and indexed using Samtools v 1.16 [17]. Aligned data were directly explored using the Integrative Genomics Viewer - IGV [17]. Reference and mutated sequence counts were extracted with custom scripts.

Copy number analysis

DNA material from BM at the time of diagnosis was used for Copy Number Alteration (CNA) analysis by multiplex ligation-dependent probe amplification (MLPA) (Mrc Holland) or the Affymetrix Cytogenetics Whole Genome 2.7M Array or Cytoscan- HD (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol.

Analysis of the B-cell repertoire by flow cytometry

Peripheral blood samples were collected from the 2 *PAX5* c.548delG carriers and 4 healthy donors. Whole blood samples were prepared for flow cytometry and analyzed according to the EuroFlow Bulk Lysis protocol [18]. PB samples were stained with a 9-color panel, and all fluorescence-conjugated antibodies are listed in supplemental Table III. Data was acquired using a Cytek Aurora (Cytek® Biosciences) and flow cytometric analysis was performed using Infinicyt (Cytognos) and DIVA software. Statistical analysis was performed by Graphpad Prism software ver.9.2.0, using t-test.

Results

We identified a novel heterozygous germline variant in PAX5 shared by two siblings with BCP-ALL; the variant - a frameshift deletion resulting in a truncated protein (NM_016734.2 PAX5 c.548delG, p.Gly183AlafsTer84, MAF 0) - was classified as likely pathogenic according to ACMG guidelines. No other known common pathogenic or likely pathogenic variants in cancer predisposition genes were identified through WES.

Familiar segregation analysis revealed a paternal origin of the *PAX5* germline variant (father and grandmother of the patients) and demonstrated an incomplete penetrance of the phenotype; the mother proved to be wild type. A healthy brother of the subjects (24 years old) was not tested for the variant because of a family decision (Fig. 1).
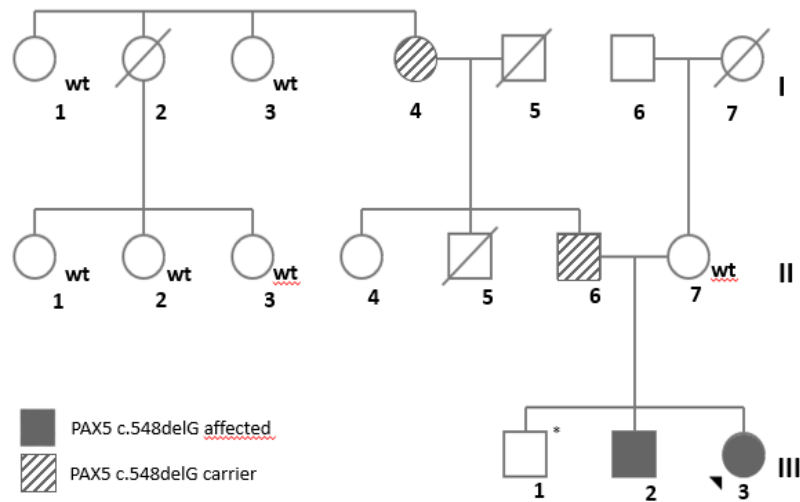
*Figure 1: Familial segregation of PAX5 p.Gly183AlafsTer84. The germline variant in the two siblings was demonstrated to be inherited from the father (II.6) and the grandmother (I,4) both asymptomatic carriers with no history of cancer. *: non tested*

In contrast to previously described *PAX5* germline families [9-14], our patients exhibit a germline frameshift variant that causes a premature arrest of the protein synthesis, suggesting a more destructive effect on PAX5 activity.

As expected, our patients exhibited an additional somatic event on *PAX5* that further impaired PAX5 function and led to the development of the leukemic clone. Strikingly, the disease samples from both siblings carried an identical additional somatic pathogenic *PAX5*

p.Pro80Arg variant (c.239C > G, p.Pro80Arg; variant fraction: 29% in Case A and 38% in Case B) (Fig. 2), which has been functionally characterized as a pathogenic variant promoting leukemogenesis [3,5].
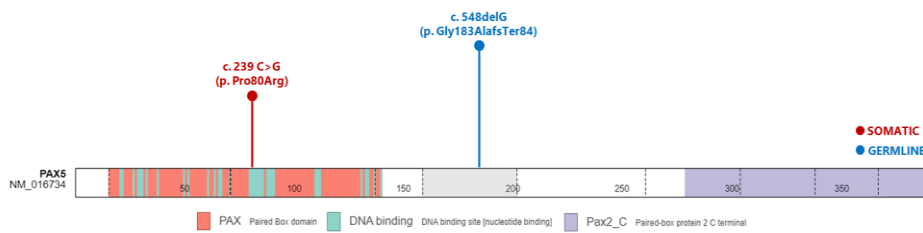
Figure 2: Graphical representation of the two identified PAX5 variants. In red: somatic PAX5 c.239C>G, p.Pro80Arg variant; in blue: c.548delG, p.Gly183AlafsTer84. The germline variant resides in the Octapeptide domain (aa 179-186 domain). The somatic variant is in the Paired box domain. Adapted from St. Jude Pecan database (Pecan database, https://pecan.stjude.cloud/PAX5).

The digital MLPA analysis excluded the presence of common CNV and, in particular, did not identify CNV alterations affecting the *PAX5* locus. Specifically, Case A showed a homozygous deletion in *CDKN2A* (exones 1-4) and *CDKN2B* (exones 1-2), whereas case B showed a heterozygous deletion on *IKZF1* (exones 1-8). This evidence is in contrast with what observed in the other PAX5 mutated families that share a 9p deletion as a second hit [9-14].

We, then, performed a nanopore long reads sequencing of the disease sample to determine whether the identified variants were in *cis* or in *trans*. The sequencing data proved that the two variants were in trans

(Reads carrying p.Pro80Arg mutation in cis with allele p.Gly183 wild-type: 96%), meaning that in the leukemic cells, both *PAX5* alleles are impaired (Fig. 3).
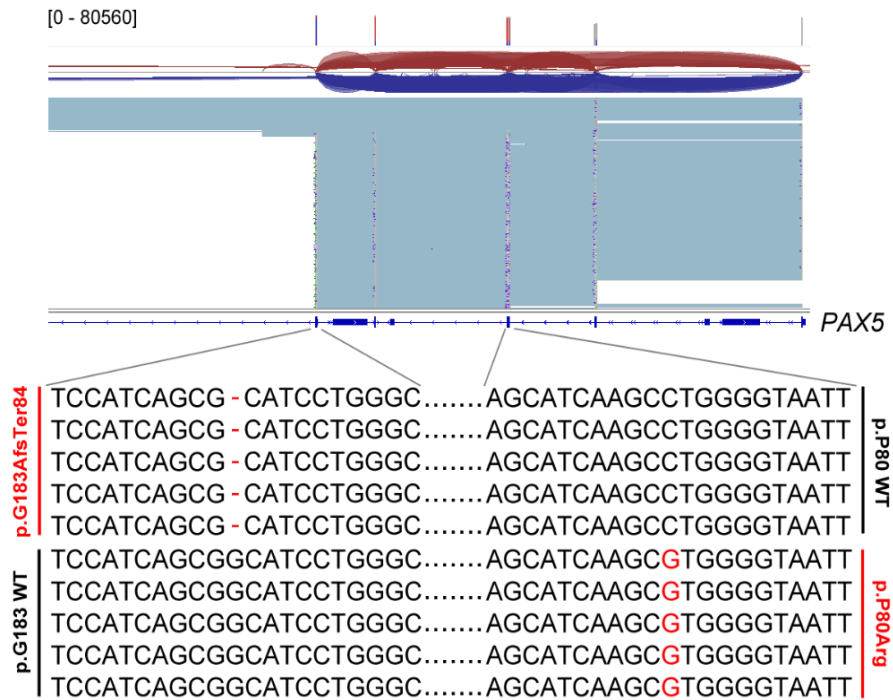

Figure 3: Nanopore long read sequencing proved that the two identified variants are in trans, as shown in the image. The sequence variants are highlighted in red.

Cumulative read depth is shown on top of the figure; Nanopore sequence reads were aligned to the human GRCh38/hg38 reference.

The concept of biallelic *PAX5* modification in the development of leukemia finds reinforcement through murine models, either Pax5+/− mice or heterozygous Pax5P80R/+ knock-in mice: in both scenarios,

the emergence of leukemia there is concurrent to the deletion or mutation of the wild-type *PAX5* allele [18,19].

Finally, to investigate a possible impairment of PAX5 function in the development of the B-cell lineage, we examined the B-cell repertoire on fresh peripheral blood mononuclear cells by FACS analysis in both healthy carriers (n=2), compared to healthy controls (n=4); interestingly, both healthy carriers had a moderate reduction in memory B cells IgD- CD27+ (healthy carriers 9% vs 27% healthy donor) (Fig. 4), while no difference was observed in the amount of B-cells (CD19+ B cells) and naive B cells (IgD + CD27-).
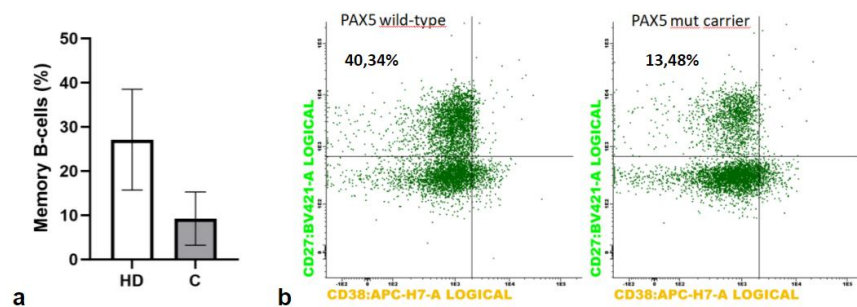
Figure 4a: B-cell repertoire on fresh peripheral blood mononuclear analysis showed a reduction in memory B-cells IgD- CD27+; (p = 0,07) Fig. 4B shows the citoflowrimetry dot plot of a PAX5 wild-type healthy donor compared with a PAX5 mutated carrier.

This evidence reinforces the hypothesis that PAX5mut healthy carriers show an impaired B-cells differentiation, since we observed a reduction of memory b-cells in the healthy carriers compared to healthy donors. Nonetheless, the two PAX5mut healthy carriers did

not have a history of increased susceptibility to infection. These findings recapitulate the impairment in B-cell maturation observed by Escudero and colleagues in PAX5 p.Gly183Arg germline families[13].

Conclusions

This report sheds light on potentially diverse mechanisms contributing to leukemogenesis in the context of *PAX5* germline mutation. The identification and characterization of germline variants is of great interest and importance in the context of leukemia predisposition, as it has clinical implications for familial genetic counseling and contributes to the understanding of the pathogenesis of ALL.

Supplementary Materials

Table S1: List of Primers

Gene	Variant	Primer	Sequence
PAX5	p.Gly183AlafsTer84	Forward	GGGTCAGTCCTTCTCAGTGC
		Reverse	TCGTCTCTCTTGCGCTTGTT
PAX5	p.Pro80Arg	Forward	GATTTAGAGAAAAATTATCCGA

Table S1: List of primers used for Sanger sequencing validation of the germline PAX5 c.548delG, p.Gly183AlafsTer84 variant and for the RT-PCR of PAX5 c.548delG,p.Gly183AlafsTer84/PAX5 c.239C>G, p.Pro80Arg cDNA fragment.

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Chapter 5

Potential role of STAG1 mutations in genetic predisposition to childhood hematological malignancies

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and NP contributed to cell cycle analysis; SR, FA, and UF performed the bioinformatic analysis; LRB collected clinical data; CS, SR, LRB, and GF contributed to data analysis and interpretation; VM and JH reviewed data analysis and interpretation; CS, SR, GF, and GC wrote the manuscript. AB and GC supervised the study; all authors revised the paper and approved the submitted version.

Dear Editor,

Cohesin ring is a multi-protein complex that plays an essential role in a wide range of cellular processes: besides its canonical role in sister chromatids cohesion and segregation [1], the complex gives a fundamental contribution to DNA repair and maintenance of genome integrity [2], and in transcriptional regulation [3]. Cohesin genes are classified as encoding core subunits (SMC1A, SMC3, RAD21, and the paralogs STAG1/STAG2), and cohesin regulatory factors (e.g., NIPBL, HDAC8, and others) [1, 2]. Among these, *STAG1* encodes for a key subunit of the complex, essential for chromatids cohesion [1, 4].

Germline mutations of cohesins lead to Cohesinopathies [5], while recurrent somatic mutations in multiple components of the complex are known in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) [6], as well as solid tumors [7].

A correlation between Cohesinopaties and cancer predisposition has not been established yet. However, the reports of three Cornelia de Lange patients (CdLS) affected respectively by Down syndrome-like acute megakaryoblastic leukemia (AMKL) [8], acute lymphoblastic leukemia (ALL) [9] and myelodysplastic syndrome (MDS) [10], suggest

that germline mutations in Cohesins could constitute a predisposing factor to hematological disorders.

The present study aims to characterize germline Cohesins variants in pediatric patients affected by hematological diseases.

We screened 120 childhood ALL consecutive diagnoses: 107 B-ALL (89.1%), 11 T-ALL (9.2%), and 2 mixed phenotype acute leukemia cases (MPAL) (1.7%). Additionally, we sequenced 19 sporadic pediatric patients referred by our clinicians for having a familial recurrence of cancer (n = 8), syndromic features (n = 9) associated with either ALL (n = 15) or AML (n = 2); two additional cases were rare pediatric MDS.

A custom Next-Generation Sequencing panel was used, including 39 genes associated with predisposition and leukemogenesis. (Supplementary Table 1). We sequenced DNA extracted from bone marrow mononuclear cells during the disease and remission phase, the latter defined by a minimal residual disease (MRD) value below $10-4$. NGS data that support the findings of this study are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress), reference numbers E-MTAB-11757 and E-MTAB-11760.

We focused on Cohesins variants and two previously uncharacterized heterozygous variants have been identified in the *STAG1* gene.

The first STAG1 variant (Arg1167Gln) was found in a 2 years old male patient affected by BII-ALL (negative for common translocations; central nervous system negative; medium risk for MRD). He was enrolled in the AIEOP-BFM ALL2009 protocol, he experienced a late

combined relapse (BM and CNS) and underwent HSCT. The patient had no comorbidities nor syndromic stigmata.

The second variant (STAG1 Arg1187Gln) was identified in a 14 years old male patient affected by MDS with an excess of blasts (MDS-EB1), with complex karyotype (47, XY,+8, del(16)(q22)[19]/46, XY[1]), who received HSCT. The patient did not show any syndromic features.

Both *STAG1* variants are located in a highly conserved region of the gene, frequently affected by mutations known to be implicated in oncogenesis (Pecan database, https://pecan.stjude.cloud/STAG1) (Fig. S1A).

The Arg1167Gln (c.3500G>A; rs747617236) is a germline missense alteration (VAF 44.6% at diagnosis and 41.3% in remission), classified as VUS in InterVar and Varsome. It is the only variant identified, among genes included in the NGS panel.

The Arg1187Gln (c.3560G>A; rs777032446) is a germline missense variant (VAF 51.9% at diagnosis) predicted as likely pathogenic in Varsome and VUS in InterVar. We validated the germline origin by PCR and Sanger sequencing of DNA isolated from liver biopsy, collected after HSCT transplantation (Fig. S1G).

The MDS patient carried also a somatic Arg953* variant (c.2857C > T) in the paralog STAG2 gene, annotated in InterVar, Varsome, and COSMIC as pathogenic in cancer (Fig. S2).

In order to set up an in vitro model to investigate the role in predisposition of STAG1 germline mutations, two Lymphoblastoid Cell Lines (LCLs) were generated through the immortalization of PB B-lymphocytes from the Arg1167Gln mutated patient (L- STAG1) and from the Arg1187Gln mutated patient (M-STAG1). As a control, four different LCLs were generated from healthy donors.

First, we confirmed by PCR and Sanger sequencing that both L-STAG1 and M-STAG1 have maintained the genetic profile of interest (Figs. S1E–H; S2C), while the CTRs' LCLs were wild type.

Second, we established the absence of other abnormalities in L-STAG1 and M-STAG1 LCLs, by karyotype and NGS custom panel analysis.

To evaluate the correlation between the different STAG variants and cancer, we analyzed the allele frequency (VAF) of the mutated positions across non-tumor and tumor cohorts. Known variants are combined and analyzed for the gene *STAG1*, transcript ID ENST00000383202. Minor allele frequencies of all coding germline variants present in *STAG1* in a global, healthy population, taken from the gnomAD database, are summed up codon-wise (Fig. S1B) and the VAF of STAG1 p.1167 and p.1187 indicates that these mutations are rare in the general population (details in Supplementary Data).

An analogous model was applied to *STAG2* R953* somatic alteration, rarely germline mutated in the non-cancer population (gnomAD database, AF < 10−5) (Fig. S2D).

To investigate the functionality of *STAG1* variants on DNA stability, we evaluated the status of chromatin exchanges during the mitotic division.

All LCLs were treated with phytohemagglutinin, to stimulate T lymphocyte growth (T0), incubated with BrdU (T24), which is incorporated only during the first mitotic division, and blocked in metaphase with Colchicine after the second generation (T72). Fluorescence microscopy after Hoechst staining showed that both M-STAG1 and L-STAG1 LCLs are characterized by a significantly higher number of abnormal chromatin exchanges. The average number of exchanges per nucleus is equal to 4.31 for L-STAG1 and 4.8 for M-STAG1, while the mean value for the four control LCLs is 3.05 (range 2.66 to 3.50; $p < 0.0001$) (Fig. 1A).

Furthermore, the population of cells that had one or more chromosomes with double exchanges is higher in both patients-derived LCLs: in L-STAG1 the percentage was 20.79%, compared to a mean of 12.66% for the control LCLs (n = 4; range 4.57 to 19.09%, $p > 0.05$), while in M-STAG1 the percentage was 22.17% ($p = 0.0069$) (Fig. 1B).

We also aimed to estimate the capability of LCLs to repair DNA after double-strand breaks (DSBs) induced by ionizing radiations. We evaluated the phosphorylation level of histone γH2AX, a common marker of DNA double-strand breaks damage [2, 11]. LCLs were seeded at different concentrations, to guarantee the exponential growth phase, they underwent X-ray irradiation at 3Gy and 6Gy and

then were marked with Phospho-Histone H2AX antibody at different timepoints of incubation (T0, T24, T48 after irradiation).

γH2AX phosphorylation status in M-STAG1 is significantly higher in basal conditions (Fig. 2A). The differential phosphorylation further increases after radiation at 3Gy or 6Gy. Fig. 2B shows a representative experiment at 3Gy, demonstrating a significantly lower capability of M-STAG1 to repair DNA after damage, compared to control LCLs.

Overall, control LCLs have a reduction of γH2AX phosphorylation at 48 h after irradiation (indicating a successful DNA repair), while M-STAG1 has an increased phosphorylation level, thus corresponding to a defective DNA repair capability.

In addition, a highly positive pH2AX subpopulation (namely pH2AX++) can be discriminated only in M-STAG1 even at basal conditions, and its level progressively increases at the time points after irradiation, thus confirming the previous result (Fig. 2C).

Similar findings have been obtained also after 6Gy irradiation (Fig. S7).

Therefore, we identified two germline variants of the *STAG1* gene in two pediatric patients, affected by B-ALL and MDS, respectively. Those variants are located in a highly conserved region where multiple variants associated with solid tumors were mapped.

For the first time, we specifically explored the functional role of germline *STAG1* variants in oncogenesis, by evaluating how they can

corrupt a pre-leukemic clone, making it genetically unstable and more prone to further somatic mutations.

We demonstrated that the STAG1-mutated LCLs have a higher number of both single and double chromatids exchanges compared to control LCLs. This is a common indicator of poor chromosomal strength and spontaneous chromosome instability, which is associated with failure of DNA repair and accumulation of DNA damage events. Similarly, higher SCE have been already found in other familial cancers, such as BRCA1/2 mutated breast cancer [12].

Moreover, M-STAG1 cells displayed an increased DNA damage sensitivity, with a significantly lower DNA repair capability after X-ray irradiation. These results are consistent with studies by Bauerschmidt et al., who demonstrated that repair of radiation-induced DNA DSBs was reduced in *SMC1-* or *RAD21*-depleted cells [13].

The germline status of the identified variants in non-syndromic patients is compatible with their effects on DNA stability and DNA damage repair mechanisms, compatible with life but predisposing to oncogenesis.

Although the preliminary evidence on *STAG1* therapeutic potential [14], further biological studies are needed before considering the clinical relevance of *STAG1* germline variants and any therapeutic translation as a preemptive intervention. Taken together, our study provides strong evidence in support of the involvement of *STAG1*

germline variants in predisposition to onco-hematological diseases in childhood.

If confirmed, cases carrying a *STAG1* germline variant would merit genetic counseling for the patient and its family, in order to make appropriate decisions for any therapeutic program (i.e., radiotherapy, selection of Hematopoietic Stem cell donor), as well as for any surveillance. It would be crucial to assess whether those variants have a de novo origin or silent carriers are present in the family.

In the future, we cannot exclude a similar scenario also involving other cohesin genes.
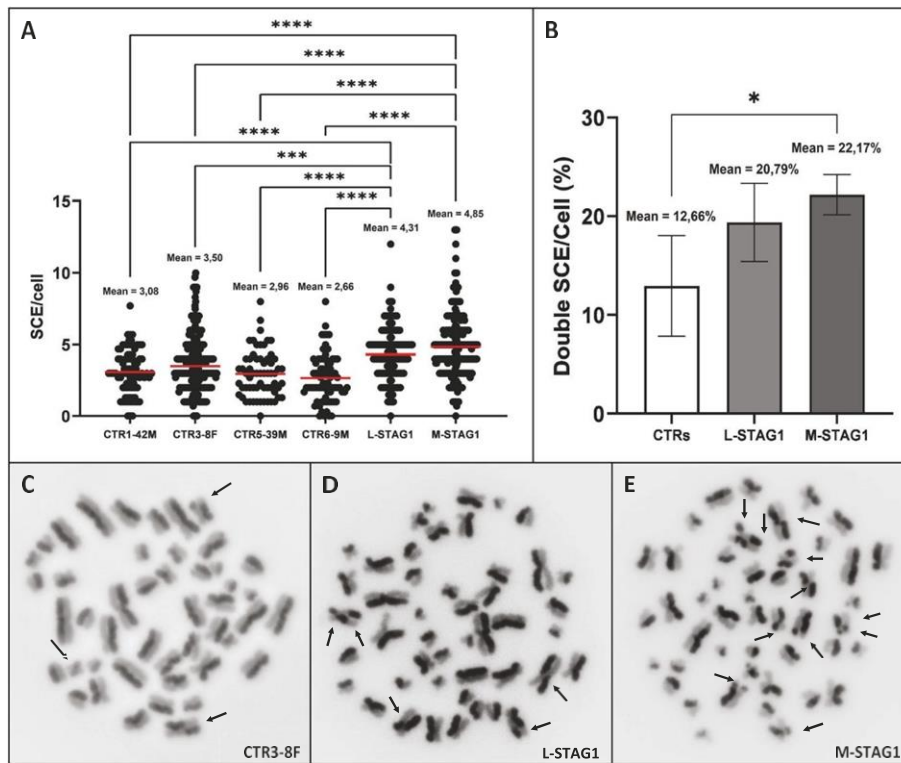
*Fig. 1 Sister chromatids exchange (SCE) incidence in LCL cells. A shows the higher number of SCE in L-STAG1 and M-STAG1 compared to control LCLs. B indicates the percentage of cells with double exchanges, which is significantly higher in M-STAG1. C–E show representative metaphases with single/double abnormal chromatid exchanges observed at fluorescence microscopy in CTR3-8F, L-STAG1, and M-STAG1, respectively. (Average of 88 metaphases for each line. Statistical analysis was performed by one-way ANOVA with Bonferroni's multiple comparison correction. * <0.05; **<0.01 ***<0.001; ****<0.0001).*
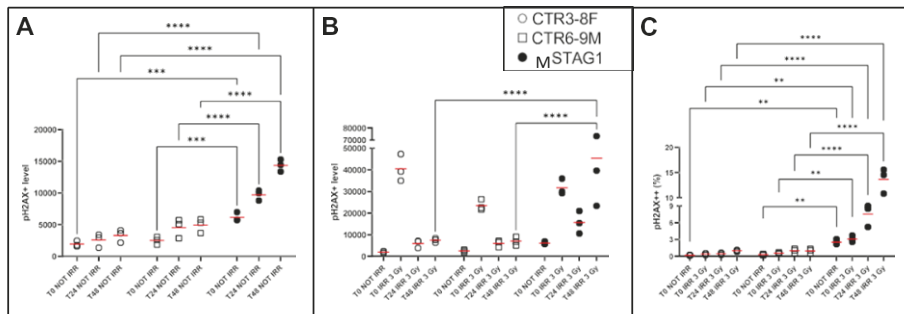
Fig. 2 γH2AX phosphorylation status before and after X-ray irradiation. Cells were seeded at different, previously established, concentrations, in order to perform the experiments in an exponential growth phase (0.1 × 106/ml for CTR3-8F, 0.22 × 106/ml for CTR6-9M and

0.18 × 106/ml for M-STAG1). A shows that γH2AX phosphorylation status of M-STAG1 is higher at basal conditions and increases during timepoints compared to control LCLs (T0: 2.8X, p < 0.001; T24: 2.7X, p < 0.0001; T48: 3.5X, p < 0.0001; MFI M-STAG1 over MFI control LCLs). γH2AX phosphorylation remains at higher levels also after irradiation [3Gy] (B, T24: 2.6X, ns; T48: 6.3X, p < 0.0001 [3Gy]; MFI M-STAG1 over MFI control LCLs, normalized on the percentage of γH2AX+ cells). The percentage of pH2AX++ subpopulation, recognized only for M-STAG1 either at basal level (10.5X, p < 0.01; percentage of pH2AX++ M-STAG1 cells over percentage of pH2AX++ control LCLs cells) or after irradiation (T0: 6.2X, p < 0.01; T24: 10.6X, p < 0.0001; T48: 14.1X, p < 0.0001; percentage of pH2AX++ cells M-STAG1 over percentage of pH2AX

++ cells controls' LCLs), shows the same trend (C). (n = 3 replicates. Statistical analysis performed by one-way Anova with Bonferroni's

multiple comparison correction. * <0.05; **<0.01 ***<0.001; ****<0.0001).

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COMPETING INTEREST

The authors declare no competing interests.

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SUPPLEMENTARY

Supplementary materials

Ethic statement

Samples were obtained from healthy donors and patients, with a written informed consent from patients or legal representatives. The study has been conducted in accordance with the ethical standards of the Declaration of Helsinki and to National and International guidelines. The study is approved by the institutional review board.

DNA extraction

Blood samples' and LCLs' DNA extractions were performed using the Wizard SV Genomic DNA Purification System (Promega Corp, Madison, WI, USA) according to protocol instructions.

DNA from the MDS patient's liver biopsy was extracted from 5 slices (5 micron each) of a formalin-fixed, paraffin-embedded (FFPE) liver biopsy. DNA extraction was performed using Maxwell® RSC DNA FFPE Kit (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. The sample was extracted with the automated DNA purification using the DNA IQ™ Casework Pro Kit for Maxwell® 16 (Promega Corporation, Madison, WI, USA).

Next Generation Sequencing and bioinformatic data analysis

A custom targeted Next Generation Sequencing (NGS) Nextera Flex DNA panel has been performed on bone marrow (BM) of hemato-oncological samples referred to our institution. Germline variants in Cohesin genes have been investigated both in disease and remission samples, defined by a minimal residual disease (MRD) value below 10^{-4}. Sequencing has been performed by Nextseq550 (Illumina, San Diego, CA) in 2x150 paired end. FASTQ files are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress), reference numbers E-MTAB-11757 and E-MTAB-11760.

Bioinformatic analysis was carried out by Sophia DDM software. Variants were filtered by variant fraction (VF)>5% and coverage at least 500X; Variant Allelic Fraction (VAF) in the population was set at 1%. We included pathogenic, potentially pathogenic and variants of unknown significance (VUS).

The most common databases of prediction were consulted for the interpretation of the pathogenicity, including: ClinVar, Clinical Genome, Varsome, InterVar, COSMIC. Benign/likely benign variants in all databases of prediction were excluded from the results (update April 2022).

STAG1 variants and cancer

The Arg1167Gln variant was identified in 3 cases out of 236700 individuals (GnomADv2.2.1 non-cancer; exome samples), while the Arg1187Gln variant was found in 1 case out of 147918 individuals

(GnomADv3.1.2 non-cancer, genome samples). Somatic, coding variants reported for adult cancer patients derived from COSMIC, GRCh37 Release 91 (CosmicCodingMuts.normal.vcf.gz, n = 1,443,198 samples) are similarly combined for each codon along *STAG1*. Both collected datasets are smoothed using the LOWESS algorithm (fraction: 0.06, iteration: 1) prior to plotting.

RT-PCR for variants validation

RT-PCR was performed using primers in the *STAG1* and *STAG2* mutated exons (Supplementary Table S2).

All RT-PCR reactions were performed at the following conditions: denaturation for 2' at 94°C, then thirty-five cycles of amplification (30 s at 94°C, 30 s at 60°C, 60 s at 72°C), using the Platinum SuperFi II DNA Polymerase–High-Fidelity PCR Enzyme (Life Technologies, Thermo Fisher, Carlsbad, California, United States).

Lymphoblastoid Cell Lines

Lymphoblastoid cell lines (LCLs) were derived from *in vitro* transformation and immortalization of B lymphocytes in fresh peripheral blood (PB) by Epstein Barr virus (EBV) (BioBank Service, Gaslini Hospital, Genova, Italy). All cells were tested for mycoplasma.

Cells were grown in T25 flasks in RPMI medium with 10% FBS, 1% Pen-Strep and 1% L-glutamine, in standard incubation conditions (37 °C, 5% CO_2).

Phenotype characterization

A flow cytometry antibody panel was developed to characterize LCL B-cell phenotype, including specific antibodies for B-cells, T-cells and myeloid cells markers, such as CD19 (FITC, #11-0199-42, eBioscience™), CD45 (PO, #MHCD4530, Invitrogen™, Waltham, Massachusetts, US), CD3 (Alexa700, #557943, Becton Dickinson™), CD13 (PE, #347406, Becton Dickinson™) and CD33 (PeCy7, #333952, Becton Dickinson™) in addition to the stemness marker CD34 (PerCPCy5.5, #347222, Becton Dickinson™). After 30' incubation (RT, in the dark), cells were washed and resuspended in 200 µl of PBS and analyzed with BD LSRFortessa™ X-20 Flow Cytometer, BD FACSDiva™ software (BD Biosciences) and FlowJo software (Tree Star, Inc. Ashland, OR, USA).

Cell Growth

To evaluate the growth rate, LCLs were seeded at different concentrations according to their previously established growth characteristics: 0.1×10^6/ml for CTR3-8F, 0.22×10^6/ml for CTR6-9M and $0,18 \times 10^6$/ml for M-STAG1 (MW6 plates). Cells were collected after 72, 96 and 120 hours. Live cells were counted by Trypan Blue exclusion both through Countess Automated Cell Counter (Thermo Fisher, Carlsbad, California, United States) and Burker' counting chamber at

optical microscope, in parallel, considering the mean of the counts. Detailed data in supplementary Figure S4.

Cell Cycle Assay

Basal and irradiated cells were collected in polypropylene tubes at $2x10^6$/ml. After a centrifugation (1800 rpm, 5') the pellet was resuspended on ice in 1 mL of GM saline buffer (Glucose 1.1 g/l, NaCl 8 g/l, KCl 0.4 g/l, $Na_2HPO_4.2H_2O$ 0.2 g/l, KH_2PO_4 0.15 g/l, EDTA 0.5M 0.2 g/l). 1.3 mL of 96% Ethanol were then added under stirring for each sample. The fixed samples, stored at + 4 °C or at -20 °C, were centrifuged (1200 rpm, 10') and then washed with 1 mL PBS. They were subsequently incubated at 4°C overnight in the dark with 1 mL solution of Propidium Iodide (2.5 µg/mL) and 12.5 µl of RNase (1 mg/mL). Flow cytometry analysis was executed using the BD LSRFortessa™ X-20 instrument and BD FACSDiva™ software. Cell cycle analysis was performed on at least 20000 cells for each. Cell cycle phase distribution was calculated as percentages by a Gaussian-modified method [1]. Detailed data in supplementary Figure S5.

Sister Chromatids Exchange Assay

One ml of LCLs cell culture suspension ($1x10^6$ cells in 5 ml RPMI 10% FBS at conc. $0.3x10^6$/ml) was added to 7 ml of medium and 250 µl of Phytohemagglutinin to stimulate T lymphocytes growth. After an incubation at 37°C for 24h, 80 µl of a 1 µg/µl BrdU stock solution was added, and samples were incubated at 37°C for 48h. In this condition,

cells grow and replicate, and new synthesized DNA will not be marked with BrdU, thus allowing the visualization of chromosomal exchanges. Colchicine has been added to block the mitotic spindle during the metaphase. Samples were incubated for 1.5 h and then transferred into 15 mL Falcon tubes and centrifuged at 1800 rpm (10'). Cells were resuspended in 7 mL of hypotonic solution (KCl 0.08 M) and incubated at 37°C for 15'. 1 mL of fixative solution (methyl alcohol and acetic acid in ratio 3:1) was added to the samples which were centrifuged at 1800 rpm (10') and then resuspended in 7 mL of fixative. The pellet was resuspended in 2 mL of fixative solution and smeared on a cold glass slide. Slides were stained with 10 µl of Hoechst (1:5000) each and incubated for 20' to visualize the frequency of SCE through a fluorescence microscope.

X-ray irradiation

LCLs underwent a cycle of X-ray irradiation, either at 3 Gy (190 V, 12 A, 5.5') and 6 Gy (190 V, 12 A, 11'), on the RADGIL instrument (Gilardoni SpA, Mandello del Lario, Italy).

pH2AX level evaluation

To investigate the capability of LCLs to repair DNA after double-strand breaks (DSBs) induced by an ionizing radiation, we evaluated the phosphorylation level of γH2AX, a DSB marker, by FACS analysis.

Cells were seeded in MW6 at the same concentration and conditions used for the growth curves, in order to perform the experiments in an exponentially growing phase.

Approximately 1×10^6 of basal or irradiated cells per sample were collected in FACS tubes and centrifuged at 1200 rpm (5'). Cells were resuspended in 1 mL of PBS and 2 mL of fixative solution (4.5% PFA/PBS, 3% final concentration). Samples were incubated for 10 min (RT). After a centrifugation (1200 rpm, 5') the pellet was resuspended in 3 mL of cold Ethanol 70% and vortexed briefly.

To remove ethanol, the cell pellet was washed 3 times in 3 mL of washing solution (0.5% BSA/PBS), resuspended with Phospho-Histone H2AX antibody (Alexa Fluor® 488 Conjugate - BD #9719) and incubated for 1h at RT. Samples were centrifuged at 1800 rpm for 5 minutes and resuspended in 200 µl of PBS.

Flow cytometry analysis was performed using the BD LSRFortessa ™ X-20 instrument, BD FACSDiva software and FCS Express Flow Cytometry from De Novo Software.

Statistical analysis

Results are expressed as mean values of technical replicates. All the experiments were performed at minimum in triplicate, evaluating standard deviation. This criterion was applied for both control and mutated samples.

Statistical analysis was performed by Graphpad Prism software ver.9.2.0 one-way ANOVA test with Bonferroni's multiple comparisons is shown as $*p<0.05$, $**p<0.01$, $***p<0.001$.

Supplementary results

LCL phenotype characterization

In order to assess that LCLs have maintained the B-lineage profile after EBV immortalization, a flow cytometry antibody panel was developed to characterize their phenotype.

We evaluated specific markers of hematopoietic subpopulations, including common lymphocyte markers (the pan-leukocyte hCD45 and hCD19 for B-cells and hCD3 for T-cells), myeloid markers (hCD13 and hCD33) and a stemness marker (hCD34). The results show a marked positivity against hCD45 and hCD19 antibodies, confirming the immortalization of the B-cell subpopulation. The results are comparable in all LCLs tested, derived both from healthy donors and mutated patients (Figure S3).

M-STAG1 and control LCLs growth is affected by X-ray irradiation.

To evaluate the different growth rates of LCLs, firstly, we tested different seeding cells for each line, in order to identify the best individual conditions that guarantee for each the exponential growing phase. On the bases of the results, we set up the experimental conditions to compare M-STAG1 with mean of control LCLs and we demonstrated that in basal condition the growth ratio for each timepoint over the previous one is comparable between the cell lines.

(Ratio T24/T0 1.41 for M-STAG1 over 1.50 for controls' mean, p> 0.05 n.s.; T48/T0: 1.29 for STAG1_LCL over 1.14 for controls' mean, p>0.05 n.s. One-sample T-Test). After X-ray irradiation, both M-STAG1 and control LCLs are characterized by a remarkable reduction in terms of growth capability in response to the damage stimulus, but the trend remains similar as shown in Figure S4.

M-STAG1 and control LCLs cell cycle is affected by X-ray irradiation - G2M block

To assess the different distribution in cell cycle phases, we evaluated the percentage of cells in each cell cycle phase (G0/G1; S; G2/M) for each LCLs.

Basal and irradiated cells ($2x10^6$/ml for each condition) were resuspended in GM saline buffer (Glucose 1.1 g/l, NaCl 8 g/l, KCl 0.4 g/l, Na2HPO4.2H2O 0.2 g/l, KH2PO4 0.15 g/l, EDTA 0.5M 0.2 g/l), fixed with 96% Ethanol and incubated with propidium iodide (2.5 µg/mL) and 12.5 µl of RNase (1 mg/mL). Flow cytometry analysis was executed using the BD LSRFortessa™ X-20 instrument and BD FACSDiva™ software. Cell cycle analysis was performed on at least 20000 cells for each. Cell cycle phase distribution was calculated as percentages by a Gaussian-modified method [1].

We didn't appreciate any significant difference between CTRs and M-STAG1 in basal condition. This trend is comparable with the growth rate observed in cell growth curves.

Only when referring to timepoints 24h and 48h, we found a slight difference in G0/G1 and S phases in M-STAG1 compared to CTRs, where mutated cells seem to have a higher percentage of cells in S phase. This trend does not persist in the ulterior timepoints.

After X-ray irradiation, cell cycle perturbations are comparable across LCLs lines. As shown in figure S5, a G2M block induced by 3 Gy and 6 Gy was detected both in control LCLs and M-STAG1.

<u>Defective capability of M-STAG1 to repair DNA after an ionizing radiation at 6Gy</u>

The γH2AX phosphorylation status of M-STAG1 remains at higher levels than control LCLs also after a higher ionizing radiation [6Gy] (T24: 1.8X, ns; T48: 7.3X, $p<0.0001$ [6Gy]; MFI M-STAG1 over MFI controls' LCLs normalized on the percentage of γH2AX+ cells) (Figure S7, panel A). These data confirmed a significantly lower capability of M-STAG1 to repair after a DNA damage, compared to controls' LCLs. Moreover, they revealed that more intense X-ray dosage causes a higher DNA damage, thus mutated cells are more impaired in repairing.

The percentage of pH2AX++ subpopulation in M-STAG1 is characterized by the same trend. (T24: 8.8X, $p <0.0001$; T48: 15.2X, $p <0.0001$; percentage of pH2AX++ cells M-STAG1 over percentage of pH2AX++ cells controls' LCLs) (Figure S7, panel B).

Figure Legend
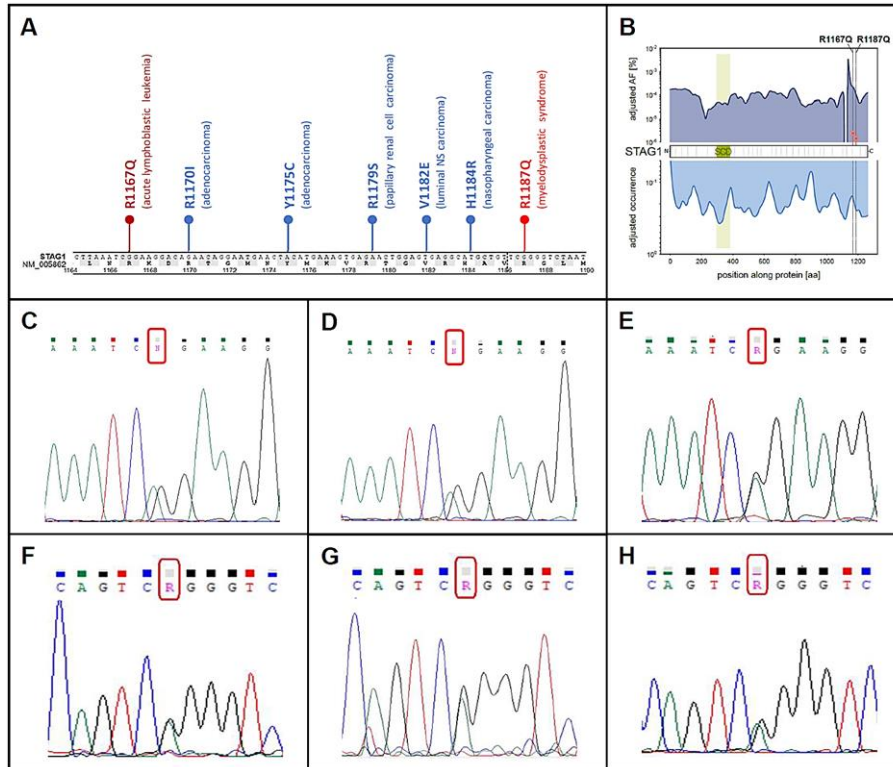
Figure S1. Validation and analysis of STAG1 variants.

In panel A, the germline ALL-mutation (Arg1167Gln/R1167Q; bordeaux label), the germline MDS-mutation (Arg1187Gln/R1187Q; red label) and the other somatic variants previously described (blue label) on STAG1. In panel B distribution of variants frequencies along STAG1, based on two databases: the top shows the adjusted AF (%) of variants in the gnomAD non-cancer database, while the bottom shows the adjusted frequency of variants in the COSMIC (somatic cancer mutations) database. In panels C-D-E, chromatograms of bone marrow ALL patient at diagnosis, bone marrow sample at remission

phase and L-STAG1, respectively. In panels F-G-H, chromatograms of bone marrow MDS patient at diagnosis, sample from liver biopsy and M-STAG1, respectively.
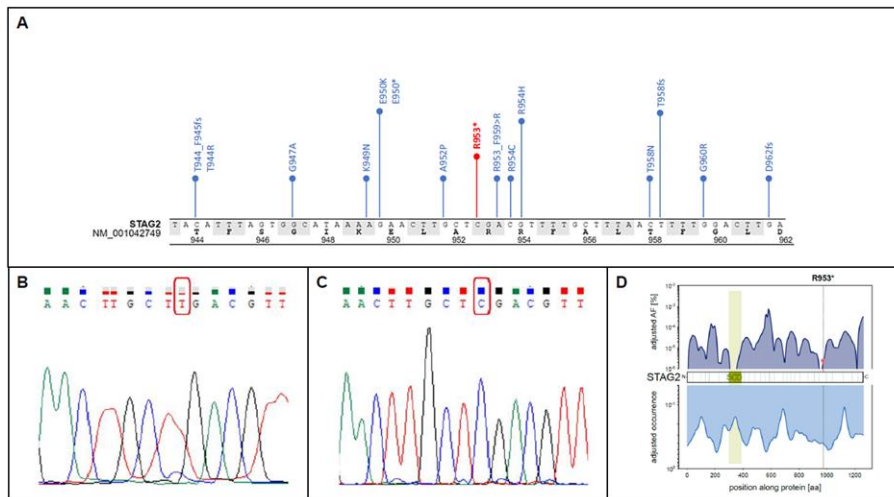
Figure S2. Validation and analysis of STAG2 variant.

In panel A, somatic STAG2 variant (Arg953*/R953*; red label) of MDS patient. In panels B-C, chromatograms of bone marrow MDS patient at diagnosis and M-STAG1, respectively. In panel D distribution of variants frequencies along STAG1, based on two databases: the top shows the adjusted VAF (%) of variants in the gnomAD non-cancer database, while the bottom shows the adjusted frequency of variants in the COSMIC (somatic cancer mutations) database.
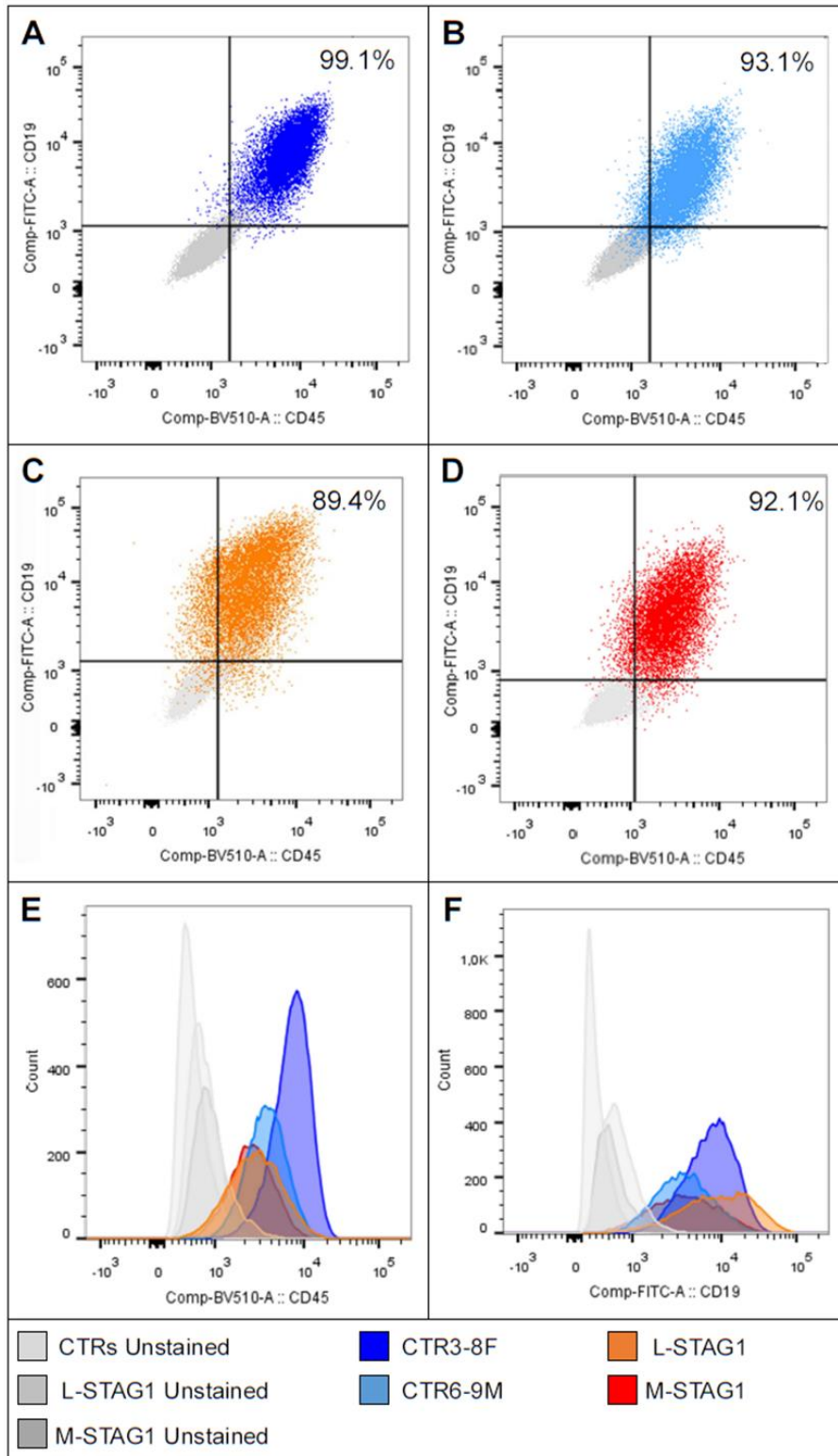
Figure S3. Phenotype characterization on LCLs.

In panels A-D dotplots of hCD19+/hCD45+ cells in CTR3-8F_LCL, CTR6-9ML, L-STAG1 and M-STAG1, respectively. In panels E-F are represented overlay histograms of hCD45+ and hCD19+ cells in each LCL, compared to unstained LCLs.
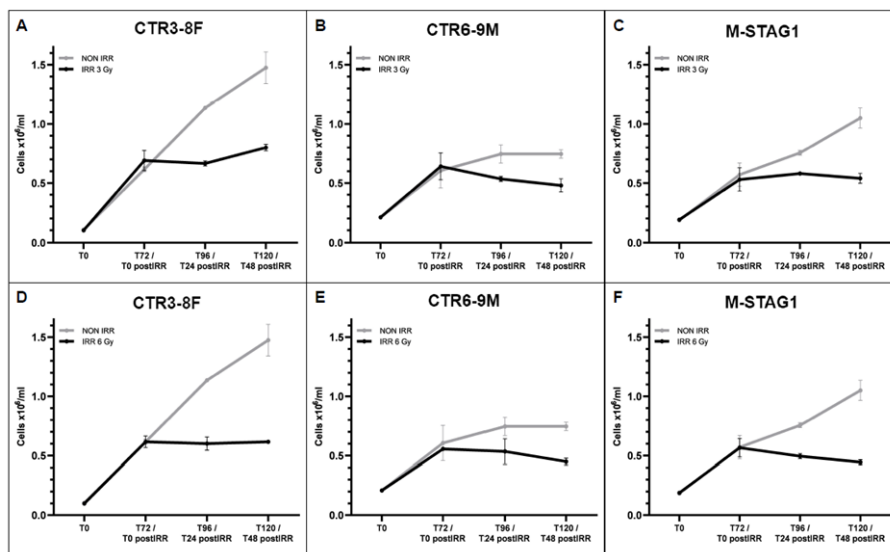
Figure S4. Growth curves of LCLs, before and after X-ray irradiation.

The reduction of cells growth rate is comparable between M-STAG1 and the mean of the two control LCLs, either after a 3Gy irradiation (A-B-C) or after a 6Gy irradiation (D-E-F).(Ratio T24/T0 0.99 for M-STAG1 over 0.96 for controls' mean, p> 0.05 n.s.; T48/T0: 0.92 for M-STAG1 over 0.95 for controls' mean, p>0.05 n.s. [3Gy]; Ratio T24/T0 0.90 for M-STAG1 over 1.01 for controls' mean, p> 0.05 n.s.; T48/T0: 0.87 for M-STAG1 over 0.82 for controls' mean, p>0.05 n.s. [6Gy]. (Statistical

*analysis performed by One-sample T-Test. * <0,05; **<0,01 ***<0,001;*

****<0,0001)
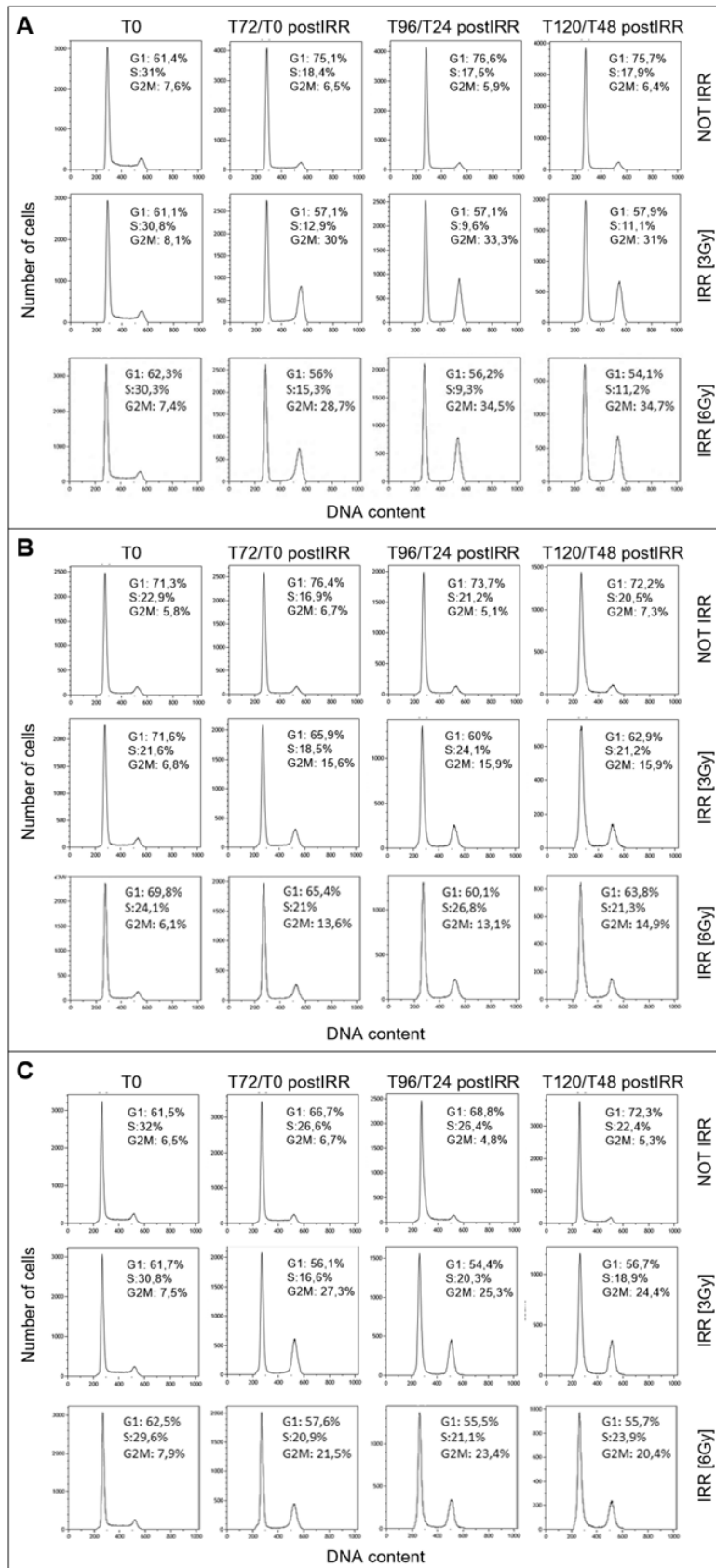
Figure S5. Effect of X-ray irradiation on the cell cycle in LCLs.

Cell cycle phase perturbations induced by [3Gy] [6Gy] irradiation on CTR3-8F_LCL (A), CTR6-9M_LCL (B) and M-STAG1 (C) after 24, 48 and 72 h after damage stimulus.
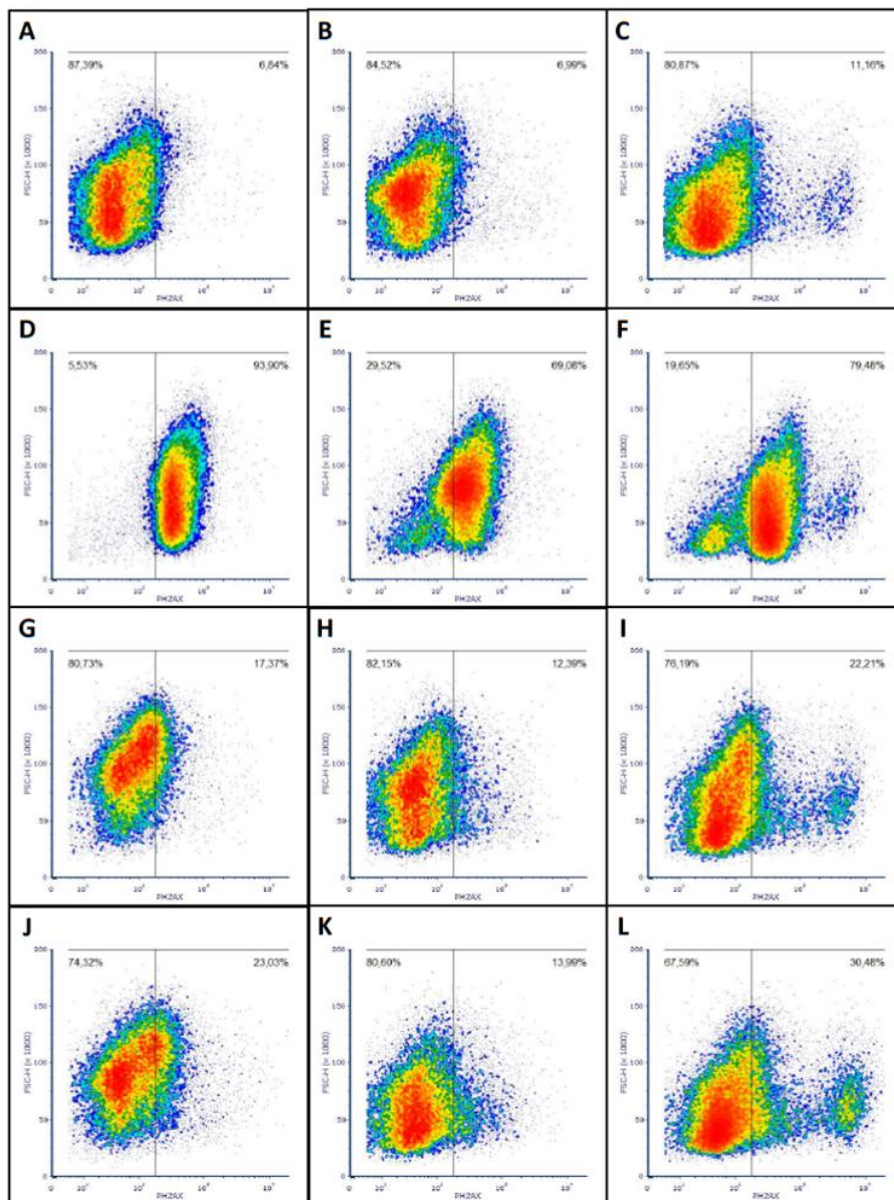
Figure S6. Representative dot plots of γH2AX phosphorylation status before and after X-ray irradiation [3Gy] – Timepoints 0h, 24h and 48h.

CTR3-8F_LCL and CTR6-9M_LCL in the first two columns and M-STAG1 in the third one. In the panels A-B-C not irradiated cells; in the panels D-E-F phosphorylation status at T0 after irradiation; in the panels G-H-I and J-K-L reduction of pH2AX+ cells at T24 and T48 can be appreciated, expression of different capability to repair after a DNA damage between control LCLs and M-STAG1.
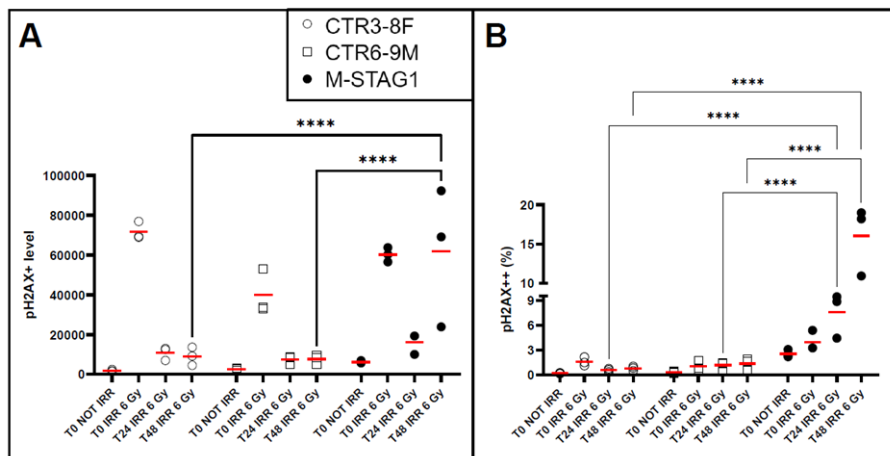
Figure S7. γH2AX phosphorylation status before and after an X-ray irradiation [6Gy]

*After a higher X-ray irradiation [6Gy], γH2AX phosphorylation status remains at higher levels in M-STAG1 compared to controls' LCLs (panel A). The percentage of pH2AX++ subpopulation (panel B) shows comparable results. (n=3 replicates. Statistical analysis performed by One-way Bonferroni's multiple comparison correction. * <0,05; **<0,01 ***<0,001; ****<0,0001)*

Tumor suppressor and DNA repair	Transcription	RAS pathway signaling	Other signaling	Cohesins	Chromatin binding and remodeling
ATM	AUTS2	BRAF	ANKRD26	BRD2	ARID5B
BLM	CEBPA	KRAS	JAK2	BRD4	CREBBP
CDKN2A	CEBPE	NF1	PIP4K2A	HDAC8	EP300
CDKN2B	ETV6	NRAS	SH2B3	NIPBL	EZH2
NBN	GATA1	PTPN11		RAD21	NSD1
TP53	GATA2			SMC1A	PHF6
	GATA3			SMC3	
	PAX5			STAG1	
	RUNX1			STAG2	

Table S1. Categories of the 39 cancer genes analyzed for mutations.

Classification of genes included in custom Next Generation Sequencing panel in different classes according to their biological functions.

Gene	Variant	Tm	Primers	Sequences
STAG1	c.3500G>A (rs747617236)	60°C	STAG1intr30-31_F	5'-AGCTAAATTCATCATCTGCTGCT-3'
			STAG1intr31-32_R	5'-CTCCATGTAACACTTACACAGCA-3'
STAG1	c.3560G>A (rs777032446)	60°C	STAG1intr31-32_F	5'-AGTCTGTAGGTCATGATTAGAAGGT-3'
			STAG1ex32_R	5'-GATCAATAACCATGGTGTCCTCAAA-3'
STAG2	c.2857C>T	60°C	STAG2intr27-28_F	5'-ACATGCTTTCTTTCTTTCCAAACAG-3'
			STAG2ex28_R	5'-GCATGGCAATGGCTTCTCTTG-3'

Table S2. STAG1 and STAG2 mutations validation RT-PCR primers.

SUPPLEMENTARY REFERENCE

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6. Summary, Conclusions and Future Perspectives

Acute lymphoblastic leukemia (ALL) is the most frequent malignancy in children[1, 2]. In the last 50 years, efforts have been spent in understanding the acquired genetic events at diagnosis and the molecular mechanisms driving leukemic transformation, clonal evolution, and relapse [1-4]. It has been for longtime a priority to focus on the identification of biomarkers with a prognostic significance to correctly stratify patients according to their specific risk[5-7]. This incredibly improved the chance of cure, which is now around 90%[2, 8].

Thus, one of the current challenges is to understand what causes ALL.

(ALL) is a complex disease with a multistep origin[3, 9-11]. It begins with genetic abnormalities that affect crucial genes involved in stem cell development and the specification of blood cell lineages during hematopoiesis. However, these initial genetic changes alone are not sufficient to trigger full-blown leukemogenesis[10, 12, 13].

Predisposition to childhood cancers is now acknowledged in at least 10% of cases, including leukemia[14-17]. In the multistep origin of ALL, the role of predisposing genetic events and the further pre-leukemic phase still need to be clarified.

In the present thesis, we focused our attention in dissecting the role of genetic predisposition in childhood acute lymphoblastic leukemia. With this purpose, we developed our study through different tasks,

characterized by the joint purpose of improving knowledge about biological mechanisms of the early stage of oncogenesis.

We started by describing the prevalence of Li-Fraumeni syndrome in an Italian cohort of childhood hypodiploid ALL. Our data confirm and reinforce what has been previously reported; by highlighting the high frequency of second malignancies in the group of patients carrying a TP53 germline variant and considering the poor EFS and OS of these subtype of childhood ALL we wanted to underlie the urgent need to take in consideration a tailored approach for this category of patients, both in term of ALL treatment[18-20] and in terms of surveillance[21, 22].

Secondly, we described the first PAX5 germline variant in a family with recurrence of BCP-ALL. Differently from what has been described up till now in PAX5-related susceptibility Leukemia[23-27], our case offers a possible new mechanism of leukemogenesis: in our family the predisposing "first" hit is a frameshift variant that leads to an important attenuation of the protein product, while the "second" hit is the well-known PAX5 p.P80R[28-30]. Our model resembles what is observed in the PAX5+/- mouse and gives new insights on the leukemogenesis process that still needs to be elucidated[31]. A single-cell NGS approach on remission samples of the two siblings could help to identify specific important pathways and altered gene suppression profiles, preceding full leukemia.

In the third chapter, we investigated the possible contribution of cohesin germline variants in the context of leukemia predisposing conditions. By screening 120 consecutive diagnoses of ALL, we found variants in cohesin genes, in a not negligible percentage of cases (data not shown). Most of them were missense, in contrast to what observed in the Cohesinopathies[32, 33], in which the most of the mutations are frameshift. Among the identified variants in the present study, we focused our attention to *STAG1* gene, due to its role in biological mechanisms fundamental for cells' integrity and survival[34, 35]. Based on our results, we can conclude that germline variants in *STAG1* lead to a poor chromosomal strength and promote spontaneous instability, resulting in a lowered response to exogenous and endogenous agents, commonly altered in oncogenesis. Moreover, the failure of DNA damage repair mechanisms worsens the accumulation of damage and aggravates the risk of somatic events, responsible of the disease' onset.

Overall, these results confirmed that germline Cohesins variants alter cellular mechanisms involved in oncogenesis even in a pre-disease phase, setting up the ideal cancer prone conditions that lead to canonical second hits. We are aware that this data contributes in a preliminary manner to a broader scenario that needs to be explored.

Dissecting leukemia predisposing conditions is fundamental because it will have an impact on different levels: 1) on *knowledge*, helping understanding the underlying mechanisms of the disease, potentially resulting in development of new treatments and therapies, as well as

the development of better screening and prevention strategies[3]; 2) on *familiar genetic counseling*, since by identifying individuals who have an increased risk of developing cancer, patients and their families clinicians will be able to take proactive steps to prevent the disease or detect it at an early stage, through appropriate surveillance protocols[36, 37]; 3) on *healthcare costs*, since by identifying individuals who are at a high risk of developing cancer and providing them with appropriate preventive measures and surveillance, we may expect a reduction in the expenses associated with cancer treatment.

However, there are still pitfalls and challenges that need to be addressed. Firstly, there is the need to consistently collect and share clinical, family history, and germline genetic data to improve the knowledge of genotype-phenotype associations, especially to dissect relationships of new variants with cancer risk[38]. Additionally, it is important to assess the functional significance of germline variants, especially of VUS, by integrating computational methods with alternative approaches, such as in vitro or in vivo experiments[16, 17].

Recognizing the growing significance of germline predisposition in the diagnosis and management of patients with hematopoietic malignancies, the World Health Organization (WHO) acknowledged the presence of myeloid neoplasms with germline predisposition in the 2016 update of the classification of hematopoietic and lymphoid tissue tumors[39]. It is likely that lymphoid neoplasms will soon receive similar recognition and that genetic counseling and germline

genetic testing will increasingly become standard practices in pediatric oncology clinics.

The information obtained from germline genetic testing will be integrated in the treatment of children with cancers, including ALL. The future challenge will be determining the most effective ways to leverage this information to further enhance overall cure rates and improve long-term outcomes for at-risk children and their families.

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