University of Pavia
Department of Molecular Medicine

PhD course in Translational Medicine
XXXV cycle

PhD thesis on

Genetic and Clinical Heterogeneity in Shwachman-Diamond Syndrome (SDS): Bioinformatic Analysis on Exome Sequencing for 16 SDS patients.

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The academic year 2022-2023

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ABSTRACT

1. Introduction

Shwachman-Diamond syndrome (SDS) is a rare autosomal recessive disease characterized by neutropenia, thrombocytopenia, pancreatic exocrine insufficiency *associated* with steatorrhea and growth failure, skeletal dysplasia with short stature, and an increased risk of bone marrow aplasia or leukemic transformation. These are the fundamental elements of the disease that must be fulfilled to meet the diagnosis of SDS. Other aspects, including hepatic complications, cardiac involvements, endocrine dysfunctions, behavioural and cognitive function abnormalities, ocular, dental, and dermal manifestations are also often observed in patients. Biallelic mutations in the *SBDS* gene, localized at 7q11.21, were found in about 90% of cases clinically diagnosed as SDS. Recently, genetic heterogeneity in SDS has been demonstrated. Biallelic mutations in *DNAJC21*, *EFL1*, or heterozygous mutations in *SRP54* have been reported also to be associated with SDS or SDS-like phenotype in a few cases.

2. Study rationale and hypothesis

The clinical spectrum of patients affected with SDS is wide. Phenotypic variability has been noticed between unrelated patients, siblings, and even within the same patient over time, making clinical diagnosis challenging in some cases. The main research hypothesis carried out in the PhD program has been addressed to explain this clinical variability by identifying any possible variants able to exert an additive or modifying effect to that produced by already known mutations in the *SBDS* gene.

3. Aims and objectives

Through this thesis work, we aimed to understand if other germline variants, in addition to the *SBDS* mutations, could explain the large clinical variability observed among the patients using the data obtained by the whole exome sequencing (WES) project for 16 SDS patients, funded by the Italian Association of Shwachman Syndrome (AISS).

The thesis work concerned several bioinformatic and molecular analysis on this type of data focusing on:

- Searching for variants in other genes related to SDS or SDS-like phenotypes, including *DNAJC21, EFL1*, and *SRP54*. We include also the *EIF6* gene since recent studies showed that *EIF6* is the most common somatic mutated gene in SDS cases, without any

evidence for myelodysplastic syndromes (MDS) or acute myeloid leukaemia (AML) development in those patients.

- The study of the distribution of variants in a panel of genes that are known to be related to haematological disorders including (MDS), (AML), neutropenia, thrombocytopenia and bone marrow hypocellularity.
- Explaining the phenotypic variability found in selected cases by filtering out the WES focusing on variants with high pathogenicity score

4. Materials and Methods

We reanalysed the available WES data for 16 Italian SDS patients, identified by their Unique Patient Number (UPN), and carrying biallelic pathogenic variants in the *SBDS* gene. All the WES data was analyzed and filtered using the Expert Variant Interpreter (eVai) platform which combines artificial intelligence with the American College of Medical Genetics (ACMG), AMP, and ClinGen guidelines to accurately report and classify genomic variants. For two SDS siblings, the VCF files were also analyzed using the digenic variant interpreter (DIVAs), an Explainable Artificial Intelligence (XAI) method for digenic variant identification and classification. Variants of interest were screened according to their position, gnomAD frequency, mutation effect, mode of inheritance, mutation database data, literature, related diseases, and *in silico* prediction tools were evaluated. The Human Phenotype Ontology (HPO) database was used to select the HPO terms describing our patients' phenotypes. Interesting variants found in the patients and their parents were validated using Sanger sequencing. Additionally, protein structural analysis was used to study the effect of the *EIF6* variant.

5. Results

The thesis findings have been explored in detail, and briefly summarized below:

- In UPN 2, a patient with a stable hematological picture, we found and confirmed a germline heterozygous missense variant (c.100T>C; p.Phe34Leu) in the *EIF6* gene. This variant, inherited from his mother, has a very low frequency, and it is predicted as pathogenic according to several *in silico* prediction tools. The protein structural analysis also envisages that the variant could reduce the binding to the nascent 60S ribosomal. These findings focused on the theory that the *EIF6* germline variant mimics the effect of somatic deletions of chromosome 20, always including the *EIF6*' s locus and that it may similarly be able to rescue the ribosomal stress and dysfunction caused

by *SBDS* mutations. This rescue may likely contribute to the stable and not severe hematological status of the proband (*These results were published in Frontiers of Genetics journal on 12th of August 2022*).

- The study of the distribution of variants in genes related to MDS and AML using a different type of bioinformatic filtering showed that UPN45, the only patient who developed MDS, had always the highest number of genetic variations that were distributed in various genetic pathways. Therefore, we postulated that in addition to the pre-existence of *SBDS* mutations, the presence of a greater number of variants in various genetic pathways could play a significant role in the development of MDS in UPN45. On the other hand, Pearson correlation testing showed a negative correlation between the absolute neutrophil count and the number of variants (R=-0.601, p=0.05) found in 16 SDS patients. We observed that the genes *VPS13B* and *CSF3R* were the most frequently mutated genes and their variants were found to be more common among patients with reduced neutrophils counts. Three out of four patients with severe neutropenia were found to be carrying biallelic rare variants in the *CSF3R* gene. *PRF1* gene missense variants were identified in all patients with severe bone marrow hypocellularity. Additionally, *VWF* variants have been noticed only in patients with severe thrombocytopenia.
- Using eVAI and DIVAs methods, we explained some phenotypic variability observed in two siblings (UPN42 and UPN43) by analysing the WES data *for both.* For UPN42, the single variant analysis did not disclose any variation which could be related to the patient phenotype. Conversely, in UPN43, we found and confirmed, using Sanger sequencing, a novel *de novo* variant (c.10663G>A, p.Gly3555Ser) in the *KMT2A* gene that is associated with autosomal-dominant Wiedemann–Steiner Syndrome. The variant is classified as pathogenic according to different *in silico* prediction tools. Interestingly, the *de novo* variants in the *KMT2A* gene were found to be associated with some of the HPO terms that only describe UPN43 including horseshoe kidney, developmental delay, bone abnormalities, expressive-language delay, minor facial dysmorphisms of hypertelorism and a wide nasal bridge and cryptorchidism. Moreover, the DIVAs method predicted the digenic combination between *KMT2A* and *SBDS* genes as pathogenic and subclassified it as a likely Dual Molecular Diagnosis. (*These findings were published in the Journal of Genes on 23 July 2022*).

6. Conclusions

The findings of this thesis underline the need of performing a comprehensive genomic analysis on SDS patients who present unexpected or unusual features. Whole exome sequencing and bioinformatics tools based on artificial intelligence are now readily available, raising the possibility that these methods may contribute to the well-known clinical variability reported with Mendelian diseases. These new data are likely, in the future, to also be relevant for personalized medicine and therapy in selected cases or groups of patients.

CHAPTER 1 - GENERAL INTRODUCTION

1.1 Shwachman-Diamond Syndrome: A General Introduction

Shwachman-Diamond syndrome (SDS) is a rare autosomal recessive disease. It was first described in 1964 by Harry Shwachman, a pediatrician specializing in cystic fibrosis, and Louis K. Diamond, a pediatric hematologist (Bodian et al., 1964; Shwachman et al., 1964). Biallelic mutations in the Shwachman-Bodian-Diamond Syndrome (*SBDS*) gene, localized at 7q11.21, are found in 90% of cases clinically diagnosed as SDS (Boocock et al., 2003). More recently, genetic heterogeneity in SDS has been demonstrated. Biallelic mutations in DNAJ homolog subfamily C member 21 (*DNAJC21*) (Alsavaf et al., 2022; Dhanraj et al., 2017), in GTPase Elongation Factor-like 1 (*EFL1*) (Stepensky et al, 2017; Tan et al., 2019), or heterozygous mutations in the signal recognition particle 54 kDa (*SRP54*) (Carapito et al., 2017; McCarthy et al., 2022) have been reported in association with SDS or SDS-like phenotype in some patients.

SDS is a multi-systemic syndrome characterized by neutropenia, pancreatic exocrine insufficiency associated with steatorrhea and growth failure, skeletal dysplasia with short stature, and an increased risk of bone marrow aplasia or leukemic transformation. These are the fundamental elements of the disease that must be fulfilled to meet the diagnosis of SDS. Other aspects, including hepatic complications, cardiac involvements, endocrine dysfunctions, behavioural and cognitive functions abnormalities, ocular, dental, and dermal manifestations are also often observed in patients (Aggett et al., 1980; Bogusz-Wójcik et al., 2021; Dror et al., 2001; Dror, 2005; Dror et al., 2011; Ginzberg et al., 1999; Kerr et al., 2010; A. S. Nelson and Myers, 2018).

The incidence of SDS was estimated to be 1/168,000 (Minelli et al., 2012), with a ratio of 1.7:1 (males to females), and no ethnic predilection (Dror and Freedman, 2002; Ginzberg et al., 1999). The median age of the disease presentation is 1.5 months, while the median age of diagnosis is 17 months. The crude mortality rate (number of deaths per year per 1000 people) is 6%. The mortality rate is highest before the age of five and after the age of 30 years (Donadieu et al., 2012; Hashmi et al., 2011).

1.2 Clinical presentations

The clinical spectrum of patients affected with SDS is widely broad and varied (Thompson et al., 2022). The phenotypic variability has been noticed between the patients, siblings, and even within the same patient over time making the clinical diagnosis challenging in some cases (Burroughs et al., 2009; Myers et al., 2014). The majority of patients have the SDS hallmark features of neutropenia, developmental delay, pancreatic insufficiency and skeletal abnormalities (A. Nelson and Myers, 2018). These features and others will be discussed in this chapter.

1.2.1 Bone marrow dysfunction and hematological manifestations

The hematological abnormalities among SDS patients are very common and may range from neutropenia to severe myelodysplastic syndromes (MDS). Hematological issues are considered the major concern of mortality among SDS patients, caused by bone marrow dysfunction (Dror et al., 2011; Furutani et al., 2022).

The hematological features among SDS could be classified into three major groups include cytopenias (neutropenia, anemia, thrombocytopenia, and lymphopenia), hematological abnormalities (macrocytic red blood cells, elevated fetal hemoglobin, and hypocellular bone marrow), and hematological complications (aplastic anemia, clonal bone marrow cytogenetic abnormalities, MDS, and acute myeloid leukemia) (Dror, 2005; Dror et al., 2011; Nelson and Myers, 1993).

Neutropenia: is the most frequent cytopenia, defined as a neutrophil count less than 1,500 × 10^9 /L. Studies reported that 88–100% of patients with SDS have neutropenia, (Burroughs et al., 2009; Ginzberg et al., 1999). About 1/3 of the patients have chronic neutropenia, and the remaining have intermittent neutropenia making them more vulnerable to developing recurrent bacterial, viral, and fungal infections due to a deficiency in the number and function of neutrophils (Smith et al., 1996). Neutropenia in SDS can develop at any age but is usually diagnosed in newborns (Dror et al., 2011).

Anemia, with hemoglobin concentration below the normal range for age, is the second most common type of cytopenia seen in SDS, reported in 42-82% of SDS patients, and it is usually asymptomatic (Ginzberg et al., 1999; Hashmi et al., 2011; Myers et al., 2014). The majority of SDS patients show mild to moderate rather than severe anemia (Dror et al., 2011; Dror and Freedman, 2002; Hashmi et al., 2011). Furthermore, about 60% of SDS patients presented with anemia, accompanied by high mean corpuscular volume (MCV) of RBCs (Hashmi et al., 2011).

Macrocytic red blood cells are described as red blood cells (RBCs) that are larger than normal. Studies found that 60% of SDS patients have macrocytic RBCs, in particular, after the first year of age (Dror et al., 2011; Hashmi et al., 2011).

Fetal hemoglobin levels, Similar to patients with other marrow failure syndromes, about 80% of patients were reported to have elevated levels of fetal hemoglobin after the first year of age (Dror et al., 2011; Hashmi et al., 2011; Smith et al., 1996).

Thrombocytopenia, platelet count <150 × 10^9 /L, is the least commonly observed cytopenia in SDS that can lead to fatal bleeding and be reported in 24%–88% of the patients. Although it is usually mild (platelet count between 100-150×10^9 /L), easy bruising has been reported in some cases, as well as fatal bleeding in cases of moderate to severe thrombocytopenia (platelet count between 10-100 or below 20×10^9 /L).

Hypocellular bone marrow (BM), is defined as cellularity below 25% or cellularity below 50% combined with hematopoietic cells below 30%. Most SDS patients show varying degrees of hypocellularity, maturation delay, fatty infiltration of the BM cells for age, and arrest in the myeloid lineage including reduced megakaryopoiesis, or erythropoiesis as well (Dror et al., 2011; Ginzberg et al., 1999; Hashmi et al., 2011; Myers et al., 2014; A. S. Nelson and Myers, 2018).

Aplastic anemia, SDS patients can present with more than one cytopenia. About 24% of them show lineage cytopenia, while 19-24% are present with pancytopenia (Burroughs et al., 2009; Ginzberg et al., 1999; Hashmi et al., 2011). Studies show that about 15% of patients, at a median age of 3.9 years, can progress to aplastic anemia which is defined as the lineage or trilineage cytopenia combined with hypocellular bone marrow without any fibrosis, or clonal evolution (Hashmi et al., 2011; Myers et al., 2014; Woods et al., 1981). However, transient and persistent aplastic anemias were seen in SDS patients (Dror et al., 2011; Hashmi et al., 2011).

Risk of MDS and acute myeloid leukemia (AML) evolution

Bone marrow failure syndromes such as SDS are typically associated with an increased risk of myeloid malignancy including MDS and AML (Bannon and DiNardo, 2016; A. S. Nelson and Myers, 2018). Generally, MDS and AML usually occur in older people (the median age at onset is 71 and 68 years respectively) (Bannon and DiNardo, 2016; Shallis et al., 2019). SDS patients are more prone to develop MDS and AML.

A recent study conducted by Elissa Furutani et al. showed that 17% of SDS patients have developed myeloid malignancy (MDS and AML) at a median age of 12.3 years (Furutani et

al., 2022). Whereas, Myers KC et al., found that 36.1% of patients developed MDS at the median age of 30 years (Myers et al., 2013).

Hematologic Complications with age

In 2022, Elissa Furutani et al. conducted a cohort study on a group of 153 SDS patients with confirmed biallelic *SBDS* mutations registered in the North American Shwachman-Diamond Registry or Bone Marrow Failure Registry. They evaluated the association between blood counts and age by analyzing 2146 blood counts. The study found that absolute neutrophil counts and hemoglobin (age up to 18 years) were positively associated with age. Conversely, platelet counts, marrow cellularity, hemoglobin after the age of 18 were negatively associated with age. Furthermore, no correlation between marrow cellularity and blood counts was found. The study also demonstrated that hematologic complications in SDS were the major cause of mortality (85%) (Furutani et al., 2022).

1.2.2 Gastrointestinal, pancreatic and hepatic features

After cystic fibrosis, SDS is the second most commonly observed exocrine pancreatic dysfunction, which considers one of the diagnostic hallmarks and an invariable feature of SDS caused by the absence of acinar cells, the exocrine component of the pancreas, which results in malabsorption, steatorrhea, failure to thrive, and low levels of fat-soluble vitamins A, D, K, and E. Unlike patients with cystic fibrosis, SDS patients have normal sweat chloride tests (Shwachman *et al.*, 1964; Ip *et al.*, 2002).

However, the pancreatic endocrine function is usually intact; a previous study reported some SDS patients with type I diabetes (Gana et al., 2011). Pancreatic imaging studies with ultrasonography or CT reveal small size for age, and characteristic pattern of fatty replacement of the pancreas, whereas patients with another pancreatic dysfunction (but not SDS) have a normal signal intensity of the pancreatic parenchyma (Toiviainen-Salo et al., 2008b).

SDS patients commonly present with impaired pancreatic enzyme secretion, as reflected by low serum trypsinogen levels, low serum amylase levels, and low serum pancreatic isoamylase levels (Ip et al., 2002; A. S. Nelson and Myers, 2018).

Trypsinogen is usually low in patients who are younger than three years of age, then becomes in the normal range in older patients, whereas serum pancreatic isoamylase remains at low levels in SDS patients of all ages (Ip et al., 2002).

However, for unclear reasons, symptoms of pancreatic insufficiency spontaneously improve with increasing age in at least 50% of patients who become pancreatic-sufficient and able to absorb fat normally (Dror and Freedman, 2002) (Ginzberg et al., 1999).

In addition to the pancreatic dysfunction features, low levels of fecal elastase were observed, varying degrees of duodenal inflammatory enteropathic features were seen in more than 50% of SDS patients (Shah et al., 2010), hepatomegaly was seen in about 15% of the patients. Elevated liver aminotransferase enzymes are observed in up to 75% of infants and children. Pathologic liver evaluations have also shown severe panlobular fatty changes, varying degrees of hepatic fibrosis and cirrhosis, and liver microvesicular and macrovesicular steatosis in several patients (Aggett et al., 1980; Brueton et al., 1977; Camacho et al., 2019; Ginzberg et al., 1999; Mack et al., 1996). However, the liver features tend to improve over time without apparent long-term sequelae and require minimal clinical attention (Aggett et al., 1980; Ginzberg et al., 1999; Ritchie et al., 2002). Note that liver complications have occurred in older patients after bone marrow transplantation (Ritchie et al., 2002).

1.2.3 Skeletal abnormalities

Skeletal defects are commonly reported in SDS patients with variable degrees of manifestations. It is usually symmetrical, varies among individuals, and over time, can be widespread or localized, usually occurring in the lower limbs than the upper limbs. In some cases, the findings may be subclinical. (Levin et al., 2015; Mäkitie et al., 2004). Growth plate abnormalities are the primary skeletal defects seen in SDS patients, in particular, the metaphyses.

Metaphyseal dysostosis has been reported in about 50% of patients in early childhood, it is usually asymptomatic, tends to become worse with age, and most commonly involves the femoral head followed by progressive thickening and irregularity of the growth plates(Aggett et al., 1980; Dror et al., 2011; Levin et al., 2015; Mäkitie et al., 2004).

In addition to the metaphyses, features of delayed appearance of secondary ossification centers, increased osteoporosis with low turnover characterized by low bone mass and bone turnover, consequently leading to vertebral fragility and fractures, rib-cage abnormalities that might lead to thoracic dystrophy during the newborn period, shortened and narrowed ribs with flared ends, digit abnormalities (clinodactyly, syndactyly, and supernumerary thumbs), and generalized osteopenia are also associated with the disease (Burroughs et al., 2009; Danks et al., 1976; Dhar and Anderton, 1994;

Ginzberg et al., 1999; Mäkitie et al., 2004; Toiviainen-Salo et al., 2008b). Other skeletal features include reduced bone mineral density, vertebral compression fractures, progressive spinal deformities (kyphosis, scoliosis, and vertebral collapse), slipped femoral epiphysis, and Vitamin D and K deficiencies have been reported in some patients with SDS (Ginzberg et al., 1999; Mäkitie et al., 2004; Toiviainen-Salo et al., 2008b).

1.2.4 Infections and immune abnormalities

Viral, bacterial, and fungal infections are common among early childhood SDS patients. Different types of infections including pneumonia, otitis media, osteomyelitis, mouth sores, septicemia, sinusitis, and skin infections have been reported (Dror et al., 2001; Ginzberg et al., 1999; Grinspan and Pikora, 2005). Neutropenia as well as defects in neutrophil chemotaxis are likely a leading factor (Aggett et al., 1979; Dror et al., 2001). A prior study demonstrated that neutrophils of SDS patients are unable to migrate and orient toward the chemoattractant gradient, which is essential for normal neutrophil function (Stepanovic et al., 2004). Another study reported an aberrant surface distribution of Concanavalin-A, reflecting a cytoskeletal defect in SDS neutrophils (Rothbaum et al., 1982). Note that, despite the low count of neutrophils, SDS patients are able to compensate sufficient neutrophils in case of infections to form abscesses (Grinspan and Pikora, 2005).

Neutropenia is not the only lineage affected by SDS. An immune function study on SDS patients has found varying lymphoid defects, including reduced numbers of B and T-cells and natural killer (NK) cells. Additionally, humoral immunity defects, including reduced serum IgG, reduced circulating B-lymphocytes, and reduced B-lymphocyte proliferation, lead to reduced antibody production. Moreover, Defects in cellular immunity included reduced circulating T-lymphocytes and reduced T-lymphocyte proliferation and, inverse CD4:CD8 ratios also have been described (Dror et al., 2001).

Regarding the Coronavirus COVID-19, Thomas J Galletta and co-workers published recently a paper describing the outcomes of SDS patients who were infected with COVID-19 in the period between May to June 2021. The results showed that the clinical course of the disease was short without any significant or major complications. In addition, the patients tolerated different types of COVID-19 vaccines without any significant issues (Galletta et al., 2022).

1.2.5 Cognitive and neurological impairments

Although cognitive function in SDS was initially reported as normal (Shwachman et al., 1964), other early studies suggested variable of cognitive impairments in some SDS patients (Aggett et al., 1980; Kerr et al., 2010; Perobelli et al., 2015).

The cognitive function of SDS patients could be impaired as indicated by lower intelligence quotient (IQ), learning difficulties, social and behavioural issues (withdrawal, somatic complaints, anxiety, depression, acting out, and impulsive, uncooperative, or aggressive behaviours) (Kent et al., 1990; Kerr et al., 2010). Both children and adults could be affected (Perobelli et al., 2015).

A case-control neuropsychological study conducted by Kerr et al. on SDS patients showed lower intellectual reasoning in children with SDS compared to their unaffected siblings and the patients with cystic fibrosis (CF). The results found that 20% of SDS patients had an intellectual disability, 20% presented severe perceptual intelligence impairment, and 12% had severe verbal intelligence. The study also showed a weakness in higher-order language skills, perceptual skills, memory, attention, academic achievement, and functional independence in SDS patients (Kerr et al., 2010).

According to Perobelli and colleagues, cognitive impairments in SDS patients are associated with diffuse brain anomalies in the grey matter and the connectivity of the white matter, which negatively correlated with cognitive performance scores. They also found that cortical thickness was greater in the patients, both right and left hemispheres, and alteration of connection fibres interfere with inter-and intra-hemispheres, which are essential for perceptual skills, verbal skills, visual-motor integration, executive functions, and memory (Perobelli et al., 2015).

Another study conducted by Myers et al. from the North American Shwachman-Diamond Syndrome Registry showed additional neurological involvements, including Chiari malformation type I, cerebellar tonsillar ectopia, and myopathy and hypotonia, which have been reported in 5% of SDS patients (Myers et al., 2014).

Recently, Akari Takai et al. provided the first direct link between a reduction in SBDS function and neurological impairments by designing a knockdown experiment for *CG8549*, the *Drosophila* orthologue of human SBDS. The knockdown was associated with locomotive disabilities, mechanically induced seizures, hyperactivity, learning impairments, and anatomical defects in presynaptic terminals (Takai et al., 2020).

1.2.6 Cardiac involvements

Cardiac features including congenital heart disease and cardiomyopathy have been reported in SDS (Hauet et al., 2013; Kopel et al., 2011; Le Gloan et al., 2014; Myers et al., 2014; Savilahti and Rapola, 1984). A study was conducted on sixteen SDS patients over 17 years. Eight of the patients have died due to cardiac failure and myocardial lesions. The autopsies demonstrated myocardial necrosis in the left ventricle of the patient (Savilahti and Rapola, 1984). Additionally, cases of cardiomegaly, atrioventricular septal defect, and dilated cardiomyopathy in patients with SDS have been also reported (Graham et al., 1980; Kopel et al., 2011; Le Gloan et al., 2014).

On the other hand, Toiviainen-Salo et al. evaluated eight SDS patients. The patients had typical myocardial structures and normal cardiac anatomy. However, subtle right ventricular (RV) diastolic function alterations at rest and depressed left ventricular (LV) contractility during exercise were observed (Toiviainen-Salo et al., 2008a). In 2015, Thomas D. Ryan and colleagues have found an abnormal circumferential strain in 33% of the patients, suggesting an association between SDS and systolic dysfunction (Ryan et al., 2015).

The French Severe Chronic Neutropenia Registry described the cardiac features found in 12 SDS patients, 6 of them had congenital heart disease (CHD), and 6 had Cardiomyopathy (CMP). The cardiac phenotypes and outcomes included coarctation of the aorta, double aortic arch, atrial septal defect, ventricular septal defect, tetralogy of Fallot, atrial septal defect, and dilated cardiomyopathy (Hauet et al., 2013).

The cardiac complications following Hematopoietic Cell Transplantation (HCT) in patients with SDS also have been described in different studies. Features of fatal pancarditis, congestive heart failure during induction chemotherapy, and long-term cardiac hypokinesia following HCT have been reported (Donadieu et al., 2005; Fleitz et al., 2002; Tsai et al., 1990).

1.2.7 Ocular manifestations

Ocular abnormalities are not very common among SDS patients (Tsilou et al., 2010). However, a few studies have found some features related to eye anomalies (Breazzano and Benegas, 2017; Dror et al., 2011; Furutani et al., 2020; Keogh et al., 2012; Myers et al., 2014; Oyarbide et al., 2016).

Two different studies by Aggett et al. and Ginzberg et al. performed a comprehensive eye examination for a cohort of SDS patients and reported occasional ocular observations, including retinitis pigmentosa, coloboma, strabismus, and punctate keratitis (Aggett et al., 1980; Ginzberg et al., 1999). A case report was published by Steven J Keogh et al. for a patient with SDS. The patient suffered from pancreatic insufficiency, bone marrow failure accompanied by trilineage dysplasia and clonal cytogenetic abnormalities, metaphyseal dysplasia, and a prolonged episode of fever due to pancytopenia. The full ocular examination revealed a perianal abscess and periorbital cellulitis in one eye (Keogh et al., 2012). Another case report for SDS patients who also developed myelodysplastic syndrome was published. The fundus examinations for the left eye revealed at least three cilioretinal arteries involving the macula, and the right fundus showed a larger and branching cilioretinal artery (Breazzano and Benegas, 2017).

Moreover, the Canadian Inherited Marrow Failure Registry presented four patients with a clinical diagnosis of SDS-like phenotype from 3 unrelated families, who were negative for mutations in *SBDS* but carried biallelic mutations in *DNAJC21*. Two of them showed ocular manifestations, in which patient 1 revealed deep-set eyes and retinitis pigmentosa. While patient 2 showed features of retinal dystrophies without pigmentation, low visual acuity, and decreased visual field (Dhanraj et al., 2017). In 2019, Shengjiang Tan et al. reported a patient with biallelic mutations in *EFL1*, the patient has severe visual impairment with low visual acuity due to high myopia. (Tan et al., 2019).

Recently, a novel phenotype related to inflammatory manifestations in patients with SDS has been described by Elissa Furutani and colleagues. They reported five subjects with SDS who developed a range of inflammatory manifestations. Three of them developed anterior segment inflammatory eye conditions, including bilateral blepharoconjunctivitis, episcleritis, bilateral keratoconjunctivitis, corneal neovascularization, and infiltrates. Additional ocular symptoms such as severe photophobia, chronic epiphora, and dry eye syndrome have been reported (Furutani et al., 2020).

1.2.8 Endocrine dysfunctions

According to Agnieszka Bogusz-Wójcik et al., endocrine dysfunctions are common among SDS patients; about 63% of the patients had at least one type of pancreatic dysfunction such as growth hormone (GH) deficiency, hypothyroidism, congenital hypopituitarism, and type I diabetes mellitus (T1DM). Moreover, SDS patients have a significantly lower height and Body Mass Index (BMI) than normal people (Bogusz-Wójcik et al., 2021).

Other studies showed additional features, including elevated thyrotropin, abnormal glucose levels, and elevated follicle-stimulating hormone level post-bone marrow transplantation (Jivani et al., 2016; Myers et al., 2013).

1.2.9 Dental and oral diseases

About 44 % of SDS patients showed dental and oral abnormalities, including delayed dental development, caries in primary and permanent dentitions, periodontal disease, recurrent oral ulcerations, and enamel hypoplasia (Ginzberg et al., 1999; Hashmi et al., 2011; Ho et al., 2007).

1.2.10 Dermatological issues

Some studies reported that patients with SDS could suffer from ichthyosis, eczematous lesions, and epidermal intracellular lipid droplets (Myers et al., 2014; Scalais et al., 2016).

1.2.11 Renal defects

A few reports showed that patients with SDS could reveal urological features such as nephrocalcinosis with preserved renal function, calcifications in the dilated renal tubule, testicular atrophy, and hypospadias. However, these features were not seen frequently among SDS patients (Myers et al., 2014; Smith, 2002; Topa et al., 2016).

1.2.12 Solid tumors

Solid tumors have rarely been reported in SDS. Six types of solid tumors have been reported in some patients affected with SDS including ovarian cancer, dermatofibrosarcoma protuberans, breast ductal adenocarcinoma, pancreatic adenocarcinoma, esophageal squamous cell carcinoma, and papillary carcinoma of the peritoneum (Alter et al., 2018; Bou Mitri et al., 2021).

1.3 Molecular aspects of Shwachman-Diamond Syndrome

1.3.1 SBDS Gene: structure, protein, expression, functions, and mutations

- **Shwachman-Diamond Syndrome *(SBDS)* Gene**:

The human *SBDS* gene is located on the long arm of chromosome 7 (7q11) with an interval of 1.9 cM; it comprises five exons spanning 7.9 kb. SBDS has a 1.6-kb transcript and encodes a protein of 250 amino acids with 28.8kDa. The gene is highly conserved with orthologs in archaea, plants, and, eukaryotes, but not in eubacteria. SBDS plays an essential role in ribosome biogenesis and RNA metabolism (Ball et al., 2009; Boocock et al., 2006, 2003; Ganapathi et al., 2007). Figure 1.1 shows the *SBDS* gene in genomic location according to Ensembl.

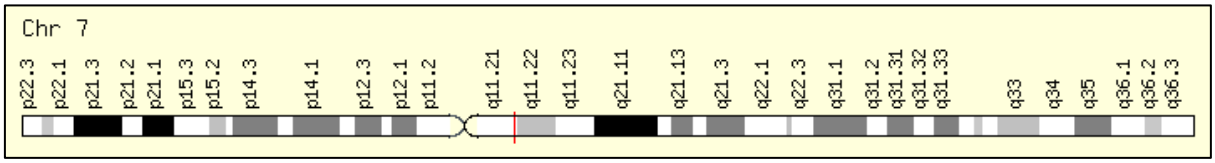


Figure 1.1. *SBDS gene in genomic location: bands according to Ensembl (https://www.ensembl.org/)*

- ***SBDS* Pseudogene *(SBDSP1)***

SBDS has an unprocessed pseudogene (*SBDSP1*), located 5.8Mb distally at the telomeric end of the *SBDS* locus, characterized by a 97% identical transcript to *SBDS*. Pseudogene *SBDSP1* contains deletions, nucleotide changes, and point mutations that could disrupt the protein-coding potential. Studies showed that about 74% of disease-associated mutations appeared to be the results of gene conversion between *SBDS* and its pseudogene at the meiosis stage; 89% of SDS patients carried at least one converted allele, and 60% carried two converted alleles. However, *SBDSP1* was identified as an oncogene with a long non-coding RNA overexpressed in human colorectal cancer cells (Boocock et al., 2003; Shi et al., 2017; Zhou et al., 2017).

➢ Sbds Protein Structure

Protein structure for Sbds by using X-ray crystallography for SBDS orthologue in archaeal ortholog *A. fulgidus* identified a three-domain architecture (Shammas et al., 2005) (Figure 1.2).

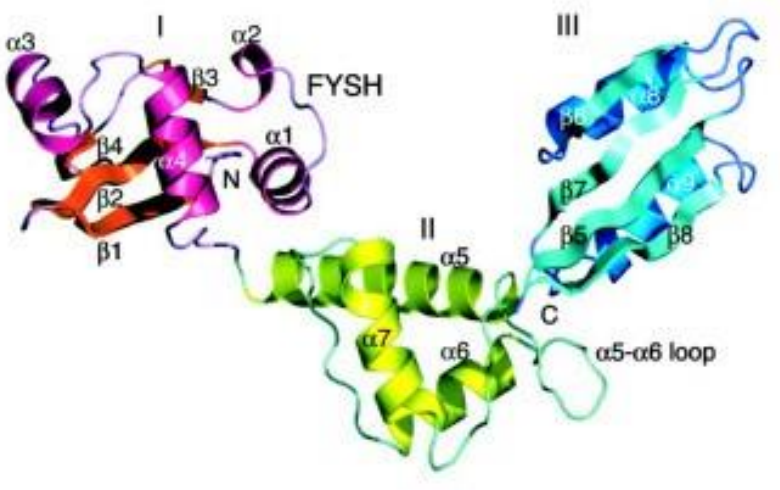


Figure 1.2. *Structure of the A. fulgidus Sbds orthologue, AF0491 (Shammas et al., 2005)* ***(I)*** *N-terminal domain FYSH (Fungal, Yhr087wp, Shwachman) spanning residues S2-S96 contains a mixture of α-helices and β-sheets in the sequence ββααββαα.* ***(II)*** *Central helical domain Spanning 13 residues D97-A170 contains three α-helices (α5,6,7).* ***(III)*** *C-terminal spanning residues H171-E250 contains a ferredoxin fold in the sequence βαββαβ.*

The N-terminal domain I (FYSH) is considered to be highly conserved and an SDS-associated mutational hotspot. This domain can functionally complement amongst closely related species. Moreover, having a high degree of dynamic flexibility can propagate structural and positional changes to other domains (Finch et al., 2011). A genetic study in *yeast* found that the central domain II and the N-terminal are sufficient for Sbds function. Whereas, C-terminal III is the least conserved domain in Sbds, identified as a common RNA-binding fold and could be an RNA recognition motif based on structural homology, and is dispensable for survival in *yeast*. The three domains are arranged in V-shape (Boocock et al., 2003; Savchenko et al., 2005; Shammas et al., 2005).

- **SBDS Protein Functions:**

- Ribosomal biogenesis by the release of eIF6

Sbds protein is nucleocytoplasmic with greater concentration in the nucleolus, consistent with its role in ribosome biogenesis. In the mammalian cells, during the final step of ribosome maturation, SBDS protein and GTPase EFL1, known as Elongation Factor Like-1, together, directly catalyzes eIF6 removal from the pre-60S ribosome subunit surface through a mechanism that requires GTP binding and hydrolysis by EFL1 (Weis et al., 2015).

eIF6 physically blocks the intersubunit bridge formation, preventing the premature joining of the two ribosomal subunits (the 60S and 40S) (Gartmann et al., 2010). The EIF6 removal, in turn, facilitates the binding of pre-60S and 40S ribosome subunits to form the mature ribosome assembly of the 80S subunit (Finch et al., 2011). In SDS, because of the loss of SBDS activity, eIF6 remains bound to the 60S subunit and prevents it's joining to the 40S subunit, resulting in decreased ribosomal subunit joining and reduced translation efficiency. In turn, immature ribosomes lead to the activation of cellular senescence pathways and finally cause a global fitness defect in hematopoietic stem and progenitor cells, eventually resulting in clinical manifestations such as bone marrow failure and hematological malignancies (Burwick et al., 2012). Figure 1.3 shows the role of SBDS and EFL1 in eIF6 removal.

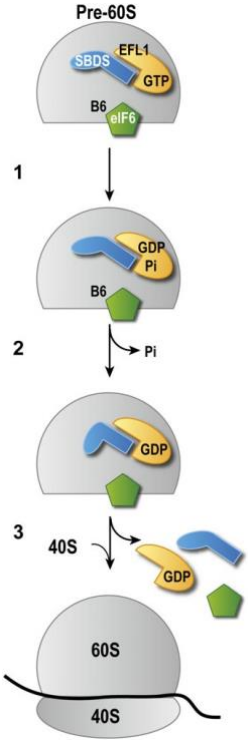


Figure 1.3. *Model of eIF6 release by SBDS and EFL1 (A. J. Finch et al., 2011).* ***(1)*** *SBDS stimulates 60S-dependent GTP hydrolysis by EFL1, generating EFL1.GDP.Pi.* ***(2)*** *Following release of inorganic Pi, EFL1 adopts its GDP-bound conformation and domain I of SBDS is rotated relative to domains II and III, directly or indirectly disrupting the inter subunit bridge B6.* ***(3)*** *Binding of eIF6 is destabilized, release of eIF6 is triggered, and EFL1.GDP and SBDS dissociate from the ribosome. Release of eIF6 allows the formation of actively translating 80S ribosomes. The putative conformations of EFL1 are not indicated.*

- **SBDS additional functions and roles**

Several studies have identified SBDS interacting proteins with functions other than ribosome biogenesis including, rRNA processing (Ganapathi et al., 2007; Moore et al., 2010; Rujkijyanont et al., 2009), microtubule stability (Austin et al., 2008), actin signaling and remodeling (Leung et al., 2011; Wessels et al., 2006), p53-mediated apoptosis and senescence (Austin et al., 2008; Tourlakis et al., 2012), mitochondrial respiration (Henson et al., 2013; Ravera et al., 2016), reactive oxygen species production (Ambekar et al., 2010), hyperactivation of mTOR and STAT3 (Bezzerri et al., 2016; Vella et al., 2020), Fas-mediated apoptosis (Ambekar et al., 2010; Rujkijyanont et al., 2009; Watanabe et al., 2009), triggering the interaction of RPL5/RPL11 with MDM2, and perturbs the MDM2–p53 interaction in case of SBDS depletion (Hao et al., 2020).

➢ *SBDS* Mutations

○ Common *SBDS* mutations

SBDS mutations are accountable in about 90% of SDS patients. Pathogenic mutations arise as a consequence of gene conversion due to recombination between *SBDS* and its pseudogene *SBDSP1*. Exon 2 in *SBDS* is considered a mutational hotspot, the most common conversion mutations are c.183_184delTAinsCT in exon 2, and 258+2T>C in the donor splice site of intron 2. c.183_184delTAinsCT is a nonsense mutation that introduces

an in-frame stop codon (p.Lys62*), whereas the mutation of 258+2T>C is a frameshift result in either an mRNA deletion with 8bp (r.251_258del) encoding a truncated protein (p.Cys84Tyrfs*4) or mRNA with exon 2 skipping (r.129_258del). Note that no homozygotes have been identified in c.183_184delTAinsCT due to the complete absence of functional SBDS protein which is incompatible with life and may lead to early lethality (Boocock et al., 2003; Nelson and Myers, 1993).

- ***SBDS* mutations among Italian SDS patients**

Aside from conversion mutations, nucleotide variations were described also in the coding region of *SBDS* leading to a rare frameshift, nonsense, and missense mutations. Table 1.1 shows 120 Italian SDS patients the percentage of each different *SBDS* mutation in one allele, considering that the other allele is always mutated with 258+2T>C mutation (Bezzerri and Cipolli, 2019).

Table 1.1. *Percentages of SBDS mutations in the 2nd allele among 120 Italian SDS patients (Bezzerri and Cipolli, 2019)*

SBDS Mutation among Italian SDS patients (2^{nd} Allele)	Percentages (%)
c.183_184delTAinsCT	56%
c.183-184TA>CT+258+2T>C	14%
c.258+2T>C	8%
c.258+533_459+403del	5%
c.212C>T	3%
c.300delAC	2%
c.92-93GC>AG	2%
c.356G>A	2%
c.107delT	2%
c.352A>G	1%
c.307-308delCA	1%
c.624+1G>C	1%
c.101A>T	1%
c. 352A>G	1%
c.187G>T	1%
c.289-292del	1%

1.3.2 Other genes associated with SDS pathogenesis

- **Elongation factor-like GTPase 1 (*EFL1*)**

In mammalian cells, SBDS corporates with GPTase EFL1 to catalyze eIF6 removal from the nascent 60S ribosomal subunit to facilitate the binding of the 40S subunit to the 60S subunit to generate the mature and functional 80S ribosome (Finch et al., 2011; Weis et al., 2015). Recently, Stepensky et al., reported that biallelic mutations in EFL1, described as an SBDS partner factor, are also associated with SDS-like phenotypes including, infantile pancytopenia, exocrine pancreatic insufficiency, and skeletal abnormalities.

The study found homozygous EFL1 mutations in four patients (siblings) from two unrelated families, the two siblings from the first family were carrying p.R1095Q and the second sibling of the second family had p.M882K mutations (Stepensky et al., 2017).

Queenie Tan et al. published a case report as further evidence for the involvement of *EFL1* in SDS-like syndrome. The report demonstrated that biallelic mutations (p.Thr127Ala) in *EFL1* cause a spectrum of symptoms including, metaphyseal irregularity, bone marrow hypocellularity, thrombocytopenia, failure to thrive, liver fibrosis, and learning disabilities (Tan et al., 2018). Moreover, Shengjiang Tan et.al, by using *in silico* and mice models, provided compelling evidence that recessive mutations in *EFL1* can cause SDS phenotypes by impairing the release of eIF6 from 60S cytoplasmic ribosomal subunits. The study reported homozygous *EFL1* mutations in 3 unrelated patients with clinical features of SDS including neutropenia, thrombocytopenia, anemia, BM hypocellularity, skeletal involvement, and failure to thrive (Tan et al., 2019).

In 2019, Jacopo Morini et al. reviewed their whole-exome sequencing (WES) data of 16 Italian SDS patients with known biallelic mutations in *SBDS*. Three heterozygous SNPs in *EFL1* (p.K711R, p.I617V, and p.L496F) in five patients were confirmed using Sanger sequencing. The structural analysis showed that the three variants cause a destabilization of the protein chain. It was not clear if the presence of *EFL1*, in addition to *SBDS* mutation, can result in a more severe picture (Morini et al., 2019).

Since the mutations in *EFL1* show evidence of symptoms related to SDS-like syndrome, OMIM has recently added *EFL1* to the causal genes of SDS, considering it the second causal gene (OMIM: 617538).

- **DNAJ homolog subfamily C member 21 (*DNAJC21*)**

DNAJC21 encodes a protein of 531 amino acids. It is associated with ribosome biogenesis by its involvement in the release of maturation factors from the pre-60S ribosomal subunit. Therefore, similar to *SBDS*, *DNAJC21* deletion in yeast leads to reduced levels of mature ribosomes and dysfunctional 60S ribosome subunit biogenesis (Qiu et al., 2006). *DNAJC21* was identified as another causal gene for the SDS-like phenotype. In 2017, Dhanraj and colleagues presented four patients with a clinical diagnosis of SDS. The molecular analysis was negative for *SBDS* mutations, but positive for biallelic mutations in *DNAJC21* (p. K34E and p.Q174Y) The patients showed bone marrow failure, short stature, failure to thrive, dental and ocular abnormalities (Dhanraj et al., 2017). Nevertheless, the DNAJC21 mutations have been recently reported as a distinct inherited bone marrow failure syndrome, with features overlapping both dyskeratosis congenital and SDS (Chirita-Emandi et al., 2022, p. 21). Morini et.al reported two Italian SDS cases carrying biallelic mutations in *SBDS* together with *DNAJC21*. Interestingly, the patients showed more severe SDS phenotypes (Morini et al., 2019). At this time, the OMIM database classified DNAJC21 as gene-related to bone marrow failure syndrome 3 (OMIM: 617048).

- **Signal Recognition Particle 54 kDa (*SRP54*)**

SRP54 is a ribonucleoprotein complex and one of the major signal recognition particles (SRPs) that associate with the ribosome during the initiation of protein translation. SRP54 mediates the cotranslational targeting of secretory and membrane proteins to the endoplasmic reticulum (ER) (Akopian et al., 2013; Keenan et al., 2001). In 2017, Raphael Carapito and colleagues reported for the first time association between *SRP54* mutations and SDS-like phenotypes. In this study, 3 unrelated patients were identified, each of whom carrying a *de novo* missense homozygous variants in *SRP54*, without any pathogenic mutations in *SBDS*. All the patients shared congenital neutropenia, short stature, and neuropsychological abnormalities. Additional features related to pancreatic dysfunction were observed in 2 patients (Carapito et al., 2017).Another study was conducted by Christine Bellanné-Chantelot et al., in which they sequenced the *SRP54* gene in 66 cases who were negative for *SBDS* mutations from the French Congenital Neutropenia Registry. Among the cases, they identified 23 mutated cases (7 familial, 16 sporadic).

Symptoms of chronic neutropenia within the first months of life were found in all patients, and additional features of SDS-like phenotype including, severe neurodevelopmental delay and an exocrine pancreatic insufficiency were observed in 5 and 3 patients respectively (Bellanné-Chantelot et al., 2018). As further evidence, a case report was published for an Italian child patient who suffered from neutropenia, recurrent infections, episodes of diarrhea, hypoplasia in the erythroid and neutrophil lineage, maturation arrest at the promyelocyte/myelocyte stage, reduced exocrine pancreatic functions. The WES identified a single *de novo* in-frame deletion in the *SRP54* gene in which the patient did not have any pathogenic variants in *SBDS* and in other neutropenia-associated genes, the report suggested the mutation in *SRP54* as a causative gene for the mentioned SDS-like phenotype (Saettini et al., 2020).

1.3.3 Clonal bone marrow cytogenetic abnormalities

Patients affected with SDS may acquire chromosomal alterations in their BM cells. Two particular types of karyotype instability have been reported including i(7)(q10) (isochromosome of the long arm of chromosome 7) and del(20)(q) (interstitial deletions of the long arm of chromosome 20) (Khan et al., 2021; Minelli et al., 2009; Pressato et al., 2012). These anomalies may remain stable, occur intermittently, and fluctuate in numbers as well (Dror and Freedman, 2002; Smith, 2002). Austin et al. suggested that spindle stability defects in SDS patients may explain the chromosomal abnormalities (Austin et al., 2008).

Studies showed that 40% of patients with SDS acquire i(7)(q10), making it the most commonly observed chromosomal anomaly in SDS. Minelli et al. demonstrated that i(7)(q10) constantly originates from the parental chromosome 7 carrying the c.258+2T>C. As it is a hypomorphic allele, this could produces, in the cells with i(7)(q10), a slightly amount of SBDS protein.(Minelli et al., 2009). On other hand, the interstitial deletion of the long arm of chromosome 20, del(20)(q) is the second most commonly observed cytogenetic abnormality in SDS and results in the deletion of EIF6 on 20q11.22. the deletion of EIF6 has been proposed to be a compensatory mechanism to restore ribosome biogenesis by circumventing the requirement for eIF6 release (Pressato et al., 2012). Interestingly, these two clonal anomalies, in the absence of additional clonal chromosomal abnormalities, are associated with a lower risk of developing

myelodysplasia (MDS) and/or acute myeloid leukemia due to partial rescue of SBDS activity or to the loss of *EIF6* gene (Khan et al., 2021, 2020; Minelli et al., 2009).

1.3.4 Somatic genetic rescue mechanism in SDS

Somatic genetic rescue (SGR) is a rare event that occurs in Mendelian disorders in which an *in vivo* somatic mutations occur in given cells. This phenomenon may partially or totally offset the effect of the pathogenic germline mutations resulting in a selective advantage over non-modified cells (Revy et al., 2019). SGR has mainly been observed in hematopoietic disorders, in which it may promote the recovery of the hematopoietic system by annulling the pathogenic effect of the germline mutations (Catto et al., 2020; McDermott et al., 2015).

The molecular mechanisms leading to SGR are variable and two SGR types have been reported: direct SGR, in which the effect of the mutation directly affects the germline mutation, and indirect SGR, which occurs when the effect modifies another gene that participates in the same pathway that is altered by the germline mutant. Importantly, the SRG phenomenon occurs at the cellular level, not on whole organisms. Thus, it is not necessarily to improve the clinical picture of the patient (Revy et al., 2019).

SGR phenomenon has been reported in several inherited diseases, including telomeropathies (Gutierrez-Rodrigues et al., 2019; Jongmans et al., 2012), Fanconi anemia (Gross et al., 2002), and Shwachman-Diamond Syndrome (Crescenzi et al., 2009; Khan et al., 2020; Pressato et al., 2012; Tan et al., 2021).

SDS is an example of the indirect SGR mechanism. Since the SDS patients are unable to produce cells with mature ribosomes and have a low translational level due to the large fraction of eIF6 that remains bound to pre-60S ribosome subunits, two SGR mechanisms were observed which provide a selective advantage over non-somatically modified cells:

I. The periodic bone marrow monitoring of SDS patients identified two common clonal chromosomes anomalies: an isochromosome of the long arm of chromosome 7, i (7)(q10), and an interstitial deletion of the long arm of chromosome 20, del(20)(q), always including *EIF6* (Pressato et al., 2015). Interestingly, these clonal anomalies, in the absence of additional clonal chromosomal abnormalities, are associated with a lower risk of developing myelodysplasia (MDS) and/or acute myeloid leukemia due to partial rescue of SBDS activity or to the loss of the *EIF6* gene (Khan et al., 2020; Minelli et al., 2009).

II. Recently in 2021, an exhaustive study on SDS patients conducted by Kennedy et al. demonstrated the frequent development of hematopoietic clones that frequently carry a heterozygous mutation in *EIF6* and *TP53* genes. The functional studies assessed a reduction in the activity of EIF6 and the corresponding improvement of the less effective SDS translation, supporting the hypothesis of increased clone fitness (Kennedy et al., 2021).

The two mechanisms are supported by a similar observation in the *yeast* model. Menne and colleagues demonstrated that missense variants in *TIF6*, eIF6 ortholog, can bypass the fitness defect resulting from *Sdo1* lacking, Sbds ortholog, through the reducing of *TIF6* binding to the 60S ribosome subunit (Menne et al., 2007).

1.4 Diagnosis of SDS

1.4.1 Diagnostic criteria

Usually, the majority of SDS patients are diagnosed at a very young age. However, in a few cases, SDS diagnosis have been reported in older children or even adults. SDS should be considered in patients with some or all of the following clinical findings (Table 1.2)

Table 1.2. *Clinical diagnostic features of Shwachman-Diamond syndrome (Dror et al., 2011; Nelson and Myers, 1993; A. S. Nelson and Myers, 2018)*

	Diagnostic tool	**Features and comments**
Family history of SDS	Family history charts	More attention should be considered especially for patients from families with a history of SDS or other bone marrow dysfunctions diseases
Pancreatic features	Pancreatic enzyme measurements, pancreatic imaging, and endoscope	○ Reduced levels of pancreatic enzyme relevant to the age: ▪ Trypsinogen (especially for patients less than 3 years). ▪ Isoamylase (especially for patients less than 3 years). ○ Low fecal elastase levels. ○ Additional features; ▪ abnormal fecal fat balance study of a 72-hour stool collection

		▪ Reduced levels of fat-soluble vitamins (A, D, E, K)
Hematological abnormalities	CBC, Immunoglobulins and lymphocyte subpopulations	Should be present on at least two occasions for at least one lineage, either intermittent or persistent; o Neutropenia (absolute neutrophil count <1500 neutrophils/mm^3). o Thrombocytopenia (platelet count <150,000 platelets/mm^3). o Anemia or macrocytosis (with hemoglobin concentration below the normal range for age, unexplained by other causes, such as iron/B12 deficiency). o Pancytopenia (Trilineage cytopenia with persistent neutropenia, thrombocytopenia, and anemia).
BM findings	BM aspirate and biopsy	o Hypocellularity. o Leukemia (particularly acute myelogenous leukemia). o Myelodysplasia without cytogenetic alterations. o Aplastic anemia without cytogenetic alterations. o Myelodysplasia and/ or aplastic anemia with cytogenetic alterations (including deletion of 20q11, monosomy 7, isochromosome 7).
Skeletal abnormalities	Bone densitometry, X-ray, and skeletal survey	o Metaphyseal chondrodysplasia (most common). o Congenital thoracic dystrophy. o Generalized osteopenia. o Spondylometaphyseal dysplasia.

		○ Additional features: ribs and joint abnormalities, low-turnover osteoporosis, vertebral compression fractures, and deficiencies in both Vitamin D and K.
Other possible findings		○ Short stature ○ Cardiac problems ○ Hepatomegaly ○ Cognition and psychological abnormalities ○ Dermal and eczematous lesions ○ Endocrine dysfunctions ○ Infections and Immune Abnormalities ○ Oral and dental diseases ○ Ocular involvements ○ Solid tumors ○ Urinary tract and renal anomalies

1.4.2 Molecular diagnosis

The diagnosis of SDS is established mainly by mutational analysis of the major gene associated with the syndrome, SBDS. If the analysis doesn't reveal any *SBDS* pathogenic variants and the phenotype of the patient clearly suggest the features of SDS, it is recommended to include in the molecular genetic testing the other genes like *DNAJC21*, *EFL1*, and *SRP54*. Depending on the patient's phenotype, different molecular genetic testing approaches can be used including a combination of gene-targeted testing (either single-gene testing or multigene panel) and comprehensive genomic testing (whole-exome sequencing, exome array, or whole genome sequencing) (A. S. Nelson and Myers, 2018).

- ***SBDS* molecular analysis**

Brief description of the protocol used:

1. analysis of exon 2 to identify common mutations
2. If we identify two of them in the patient, we can pass to analyze the parents;

3. If we identify only one common mutation, we have to sequence all exons to identify the second mutation, probably it would be rare.

- **Comprehensive genomic testing (whole-exome sequencing, exome array, or whole genome sequencing):**

If the gene-targeted testing is not diagnostic, or the diagnosis of SDS is not considered due to the atypical phenotypic features, the performing of comprehensive genomic testing is considered the best option. The most commonly used method is WES. If WES is not diagnostic, the exome array or whole-genome sequencing (WGS) might be considered when it is clinically available (A. Nelson and Myers, 2018).

1.5 Clinical management and treatment

The clinical management of SDS requires a multidisciplinary team of different specialists from the following medical fields: clinical genetics, hematology, pediatrics, gastroenterology, orthopaedics, immunology, endocrinology, psychology, dentistry, and ophthalmology (Dror and Freedman, 2002; Furutani et al., 2020; Myers et al., 2013; A. S. Nelson and Myers, 2018; Rothbaum et al., 2002).

- **Exocrine pancreatic insufficiency**

Patients with SDS should also be followed by a gastroenterologist for the management of gastric and pancreatic manifestations of SDS. Pancreatic insufficiency can be treated with pancreatic enzyme supplements, including amylase, lipase, and protease. If enzyme replacement fails to improve absorption, an H2-receptor antagonist could be a second option (Burroughs et al., 2009; Dror et al., 2001; A. S. Nelson and Myers, 2018).

- **Bone marrow and hematological complications**

All SDS patients should be monitored by a hematologist. Recurrent bacterial, viral, or fungal infections due to neutropenia are treated with prophylactic antibiotics. In severe cases, using of granulocyte-colony stimulation factor (G-CSF) can be considered. Thrombocytopenia, bleeding episodes, or vitamin K deficiency are usually treated with tranexamic acid, aminocaproic acid, or vitamin K supplement, while, platelet transfusion may be considered in severe thrombocytopenia cases. Low hemoglobin or anemias are treated with chronic transfusion therapy and iron-chelation programs (Ambekar et al., 2010; Dror et al., 2001; A. S. Nelson and Myers, 2018). Severe pancytopenia, bone marrow transformation to myelodysplastic syndrome, or acute myeloid leukemia (AML) require hematopoietic stem cell transplantation (HSCT). Studies reported a successful HSCT of a

matched related donor, matched unrelated donor, and unrelated umbilical cord blood. The survival rate of HSCT for patients with non-malignant severe cytopenia is higher compared to those with MDS or AML (Cesaro et al., 2005).

- **Skeletal abnormalities**

Severe skeletal abnormalities in SDS patients require special orthopaedic care, for instance, severe rib and joint involvements may require surgical intervention such as corrective osteotomy. Some patients with asphyxiating thoracic dystrophy due to rib cage restriction and skeletal dysplasia will require consultation between orthopaedic surgeons and pediatric pulmonary specialists familiar with SDS. Furthermore, evaluation and treatment for those with low bone density should be implemented including adequate calcium and Vitamin D intake (Burroughs et al., 2009; Dall'oca et al., 2012; A. S. Nelson and Myers, 2018).

CHAPTER 2 – STUDY RATIONALE AND OBJECTIVES

The present thesis is part of a large Italian project on the clinical and genetic basis of SDS funded by the Italian Association of Shwachman Syndrome (AISS). The general aim of this project was to contribute to a better definition of the genetic basis, gene-phenotype correlations and pathogenic mechanisms of SDS.

2.1 Study rationale and hypothesis

The clinical spectrum of patients affected with SDS is extremely wide as described in chapter 1. Clinical diagnosis can be difficult in some cases because of phenotypic diversity that has been observed between unrelated patients, siblings, and even within the same patient over time. The main research hypothesis carried out in the PhD program has been addressed to explain this clinical variability by identifying any possible variants capable of having an additive or modifying effect on the impact caused by the known mutations in the *SBDS* gene.

2.2 Study objectives

Within this research program, the aim of this PhD project was to understand if other germline variants, in addition to the *SBDS* mutations, could explain the large clinical variability observed among the patients by reanalysis of data obtained by the WES project using a new variant interpreter (eVai) platform. The thesis work concerned several bioinformatic and molecular analysis on this type of data focusing on:

- Searching for variants in other genes related to SDS or SDS-like phenotypes, including *DNAJC21, EFL1*, *SRP54*. This work focused also on searching variants in the *EIF6* gene, since recent studies showed that *EIF6* is the most common somatic mutated gene in SDS patients whose clinical picture did not show any evidence of MDS or AML development.
- The study of the distribution of variants in a panel of genes that are known to be related to haematological disorders including MDS, AML, neutropenia, thrombocytopenia and bone marrow hypocellularity.
- Explaining the specific phenotypic variability found in two SDS siblings by filtering variants with high pathogenicity scores.

CHAPTER 3 - MATERIALS AND METHODS

3.1 Whole Exome Sequencing (WES)

3.1.1 Definition, Uses and Significance

The human genome contains about $3x10^9$ bases building coding and non-coding DNA sequences (Rabbani et al., 2014). The exome part, which contains the coding information of protein, constitutes only around 1-2% of the total genome. (Nurk et al., 2022). Several studies showed that about 85% of all disease-causing mutations in Mendelian diseases and acquired clonal diseases, such as cancer, are located in the exome part of the human genome (Stenson et al., 2009; Stranneheim and Wedell, 2016). For this reason, targeted sequencing of the inclusive coding parts (exome) has the advantage to identify the causes of a vast number of rare, particularly monogenic, diseases as well as predisposing variants in common disorders and cancers (Nakagawa and Fujita, 2018).

Explosive advances in bioinformatics and computational analyses, Next-generation sequencing (NGS) or massively parallel sequencing (MPS), including whole genome sequencing (WGS), targeted genes sequencing, RNA sequencing, and WES have allowed performing comprehensive genomic analyses at clinical and biomedical research levels (Nakagawa et al., 2015). WES technique is a comprehensive screening of nearly all the coding regions to identify mainly the base changes and could be used also to identify copy number variations of the human genome.

WES is an important tool in the fields of personalized and precision medicine because of its accuracy, efficiency and cost-effectiveness in finding novel pathogenic mutations, identifying new causative genes, and thus providing confirmation to clinical diagnosis, and treatment plan selection, which is higher when compared with other methods. At the research level, WES has become widely utilized and improved our understanding of genetic pathology and the genotype-phenotype relationships (Suwinski et al., 2019).

WES technique produces about 10 Gb of the final output, which is approximately 10-fold less than WGS. Furthermore, WES is considerably less pricey compared to WGS (Bras et al., 2012; Buermans and den Dunnen, 2014). WES is less biased than targeted exome sequencing since it does not rely on any postulation of particular genes' involvement in a given trait and phenotype (Vorsteveld et al., 2021).

3.1.2 WES in rare diseases

To date, approximately ten thousand rare diseases are known (Turro et al., 2020). Together these diseases contribute to mortality, morbidity and medical care cost significantly (Chung et al., 2020). Globally, rare diseases affect about 1 in 20 individuals, but the minority of affected patients receive a clear molecular and clinical diagnosis due to the wide phenotypic spectrum and clinical overlapping among these diseases (Boycott et al., 2017; Turro et al., 2020). Thus, establishing an accurate genetic diagnosis can reduce costs, and aid in clinical management and personalized therapies (Clark et al., 2018).

Nowadays, the presence of WES technology can be used to search for genetic variants that may explain the clinical picture of patients by using rapid large-scale sequencing, particularly the coding regions, of the patient genome. (Marshall et al., 2020). The recent development of WES, enables investigators to detect a wide range of pathogenic allele types and becoming as an effective first-tier test for patients with high diagnostic uncertainty degree, therefore, improving medical management, and eliminating the diagnostic uncertainty burdens on patients and their families by reducing the cost and time of diagnosis. (Scocchia et al., 2019). In addition to the identification and characterization of new causative genes for unknown rare diseases' genetic background, WES also has expanded the knowledge related to phenotypic variations present among patients with known syndromes (Gonzaga-Jauregui et al., 2015; Prada et al., 2014).

3.1.3 General workflow of WES

The WES method adopts a DNA library preparation technique based on hybridization and capture technology, which requires a minimal amount of initial DNA (50 ng).

The main steps of the DNA library preparation procedure are summarized here:

- Genomic DNA (gDNA) preparation through two successive dilutions to get a starting concentration between 20 to 25 ng/µL.
- gDNA fragmentation can be carried out using one of two techniques:

  - enzymatic fragmentation, when an endonuclease fragments the gDNA randomly every 300 bp;

  - mechanical fragmentation with ultrasound through the Covaris sonicator (S220 Focused-ultrasonicator, Covaris). This method is more complicated because it requires additional steps: end repair, purification with magnetic beads (Agencourt

AMPure XP reagent, Beckman Coulter), adenylation at the 3 ends, and another purification step with beads magnetism.

- Ligating adaptor sequences to each generated fragment is required for the final sequencing reaction. While it happens after mechanical fragmentation, this process takes place simultaneously with enzymatic fragmentation.
- Evaluation of the DNA library's quality and quantity by Bioanalyzer, which enables assessment of each library and evaluation of average fragment length (245-325 bp). An average peak width of less than 245 bp may indicate insufficient starting gDNA in the fragmentation reaction, which could increase the number of duplicates in the sequence. Peak average sizes greater than 325 bp, however, may indicate incorrect enzyme functionality, which could affect the accuracy of the final sequencing.
- Hybridization using a specific Capture library containing the biotin-labelled probemix specific for the genetic regions to be analyzed. The volume of the DNA library varies depending on the capture library based on the capture library. The PCR program is 95°C for 5 minutes, 65°C for 10 minutes, 65°C for 1 minute (step required for capture library addition), 60 cycles of 65°C for 1 minute and 37°C for 3 seconds, followed by final hold step at 65°C.
- Streptavidin beads (Dynabeads Myone Streptavidin T1, Thermo Fisher Scientific) protocol can detect the biotin particles used to identify the capture probes and is utilized to capture the targeted molecules. In this step, non-hybridized fragments are eliminated after agitation for 30 minutes at 1400 rpm in Thermoblock (Eppendorf).
- The second round of magnetic bead purification is performed after the collected library is amplified and a special combination of two sequence indices is added via a PCR reaction. By doing this, even when pieces from other samples are combined, each fragment of each sample will retain its identity.
- Evaluation of the library's quality and quantity. First, this was performed using a High Sensitivity kit on a Bioanalyzer. Next, quantification was carried out using a Qubit instrument (ng/L), and values were then converted to nM using the formula below:

$$[nM] = \frac{\left[\frac{ng}{\mu L}\right]}{(660 * Caputered\ library\ average\ length)} * 10\text{^}6$$

- Pooling of samples for multiplexed sequencing. The concentration of the final pool is variable (2-8 nM) and is based on the minimal concentration obtained for samples to

be pooled together. Up to 9 WES can be pooled and sequenced on an Illumina NextSeq500 sequencer.

3.1.4 WES filtering and variant prioritization of our samples

In 2015, we performed WES on 16 DNA samples obtained from blood samples of SDS patients after their acceptance to the project. Samples eligible for WES held as key features a concentration of 50 ng/ul, a ratio 260/280λ and 260/230λ, respectively, at least equal to 1.8 and 1.8–2.1, and a good integrity evaluated by agarose gel electrophoresis. Sequencing libraries of the samples were prepared using the exome enrichment kit *Agilent SureSelectXT Human All Exon V5+UTRs*. WES was performed on the HiSeq 1,000 (Illumina, 2 bp × 100 bp). The processing of sequencing data was based on the ISAAC pipeline (Raczy et al., 2013); for data alignment and for variants calling, we used, respectively, the ISAAC aligner and ISAAC variant caller. From the samples alignment we obtained a mean coverage of 65% and a coverage >20X corresponding to 91% of the complete exome sequences.

3.2 Bioinformatic analysis

For the annotation and the analysis of the variants in the variant call format (VCF) files, generated by WES for each sample, the expert variant interpreter (eVai) platform (https://www.engenome.com/evai/) (Nicora et al., 2022) was used.

3.2.1 The Expert Variant Interpreter (eVai)

The Expert Variant Interpreter (eVai) is a bioinformatics cloud-based solution. The platform of eVai was developed by enGenome S.r.l, a private bioinformatics company (www.engenome.com). By combining Artificial Intelligence with ACMG guidelines, eVai accurately classifies and prioritizes every genomic variant (SNV, INDEL and CNV), accelerates germline variant interpretation and improves the diagnostic yield by increasing efficiency and standardization. eVai automatically pre-classifies all types of variants in the VCF files according to ACMG recommendations and guidelines and scores them based on their pathogenic or benign supporting evidence.

eVai enables professionals and geneticists to collect and curate findings in their private repository, the lab knowledgebase, designed for a continuous and traceable evidence-based reassessment. It can be used for row data-driven from whole genome sequencing,

whole exome sequencing and gene panel files for single proband or even for family analysis (www.engenome.com).

3.2.1.1 eVai features and interface design

eVai used an intuitive interface designed to simplify and accelerate evidence-based evaluation, a few clicks are sufficient to identify the most relevant set of variant-disease associations and start the analysis. All variants are prioritized and ordered by pathogenicity scores thus allowing for speed-up variant evaluation and assessment. All information related to each variant is immediately accessible. Multi-tab functionality allows one to open and compare several samples in parallel (Figure 3.1).

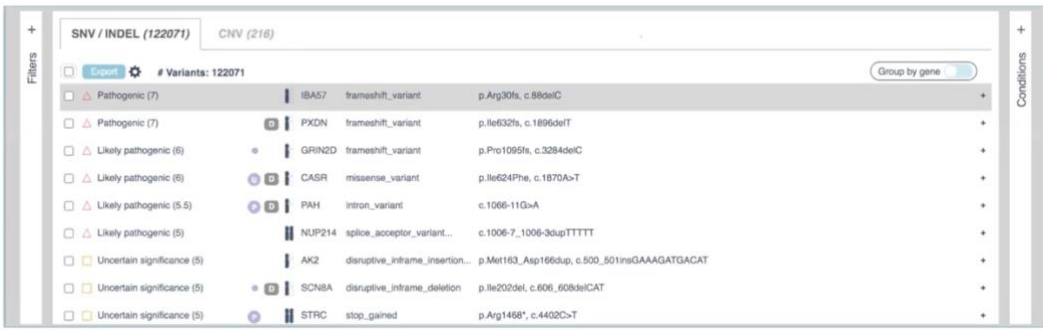


Figure 3.1. *eVai interface design*

3.2.1.2 eVai Pathogenicity score

eVai gives a pathogenicity score for every single variant calculated by enGenome benchmarking analysis conducted on more than 14,000 clinically observed variants, thus enabling variant prioritization. eVai demonstrated to reach 97.3% in sensitivity and 97.8% in its specificity (Nicora et al., 2022). The pathogenicity score for all variants, including VUS, allows investigators to focus on the most promising uncertain variants. In table 3.1 we illustrate the eVai pathogenicity scoring values and their prediction.

Table 3.1. *eVai pathogenicity score prediction*

eVai pathogenicity score	Prediction
Pathogenicity Score < 0	Variants with low or no impact on gene function
pathogenicity Score 0-2	Variants with Possible impact on gene function
pathogenicity Score 2.5-5	Variants with a high impact on gene function
pathogenicity Score ≥ 6	Variants with a very high impact on gene function

3.2.1.3 Variant filtering

By leveraging multiple omics resources, eVai allows users to filter variants based on a certain ACMG classification, pathogenicity score, allele frequency according to different population databases, ClinVar, quality metric (minimum coverage, quality, genotype information), inherence pattern, matching one or more phenotype HPO terms. It is possible also to create virtual gene panels and custom variant filters (Figure 3.2).

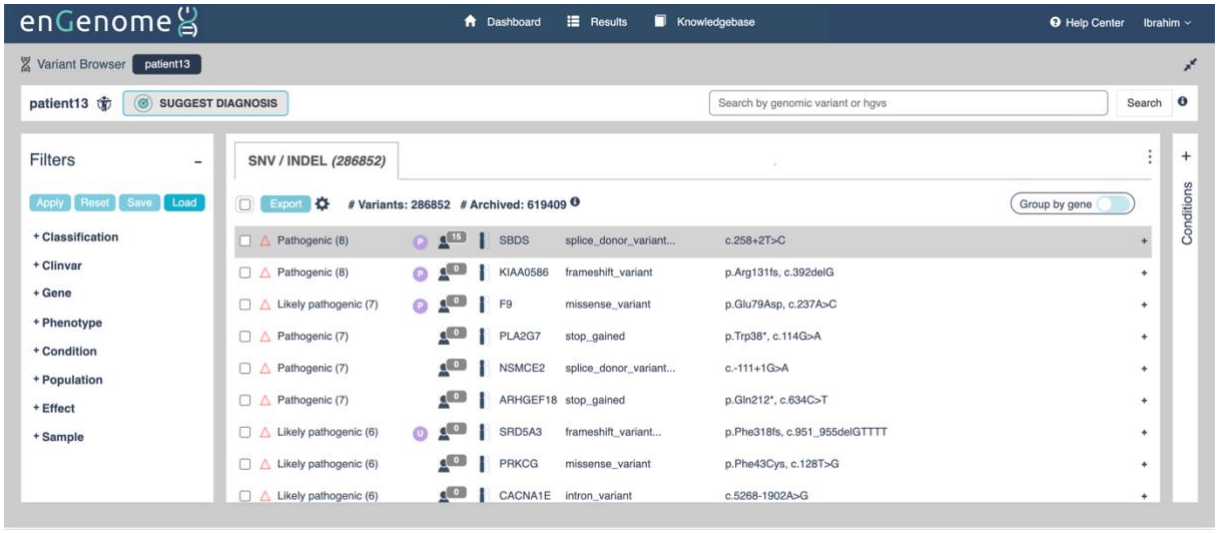


Figure 3.2. *eVai filtering options*

3.2.1.4 The digenic variant interpreter (DIVAs)

The digenic variant interpreter (DIVAs) is a machine learning-based method for digenic variant identification and classification. Briefly, this method starts from the patient's phenotypes (expressed as HPO terms) and variants (in VCF format). DIVAs generate and evaluates the list of all candidate digenic combinations.

As output, DIVAs provides a report containing the list of the digenic combination classified as pathogenic according to patient's phenotypes prioritized according to the probability of causation.

If family information is available, it can be integrated with the genetic profile of affected and not affected family members.

The digenic inheritance hypothesis was tested starting from a list of candidate variants obtained by filtering out proband's variants matching these criteria:

- Low-quality variants (QUAL<30);
- Variants with few or no impact on the gene (Pathogenicity Score <0);

- Deep intronic variants.

Briefly, this method, starting from the patient's phenotypes (as HPO list) and variants (in VCF format) exploit features at the variant level, at the gene level and at the phenotype level to classify digenic combination's pathogenicity. Furthermore, XAI explains acronym is used to investigate its digenic mechanism and subclassify each predicted pathogenic digenic combination as a True Digenic/Composite condition (where an interaction between the mutated genes triggers or exacerbates the phenotype), or a Dual Molecular Diagnosis (where two independent genetic events occur in the same individual causing blended phenotypes).

3.2.2 Variants interpretation tools

3.2.2.1 Allele frequency databases

- ***The Genome Aggregation Database (gnomAD)****:* is the largest and most commonly used database for population variation; it is built from a wide variety of large-scale sequencing projects of both exome and genome sequencing data. Through a global collaborative effort on data sharing, the gnomAD database collects data from over 195,000 individuals (Gudmundsson et al., 2021). More than 140 principal investigators contributed genome data from over 60 studies, as well as data from other population datasets (https://gnomad.broadinstitute.org/)

3.2.2.2 Mutation databases

- ***ClinVar****:* is a public archive of reports on the relationships between human variations and phenotypes, along with supporting evidence. ClinVar processes submissions that include variants discovered in patient samples, claims about their clinical significance, information about the submitter, and other supporting information. All Variants are mapped to reference sequences and reported in accordance with the HGVS standard (Landrum et al., 2020). ClinVar belongs to the National Institutes of Health (NIH). More than 1300 organizations including research and clinical laboratories, physicians, patients' registries, universities, and other experts, have submitted variant interpretations to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).

- ***The Human Gene Mutation Database (HGMD):*** is a comprehensive collection of germline variations in genes related to human Mendelian disorders. HGMD collects all germline disease-causing variants and disease-related / functional polymorphisms reported in the literature, research, clinical, or commercial background. HGMD Makes these data available in an easily accessible format. The database currently comprises over 289,000 different gene mutations identified in over 11,100 genes manually collected from 72,987 publications published in over 3100 peer-reviewed journals (Stenson et al., 2020) (http://www.hgmd.cf.ac.uk/ac/index.php).

3.2.2.3 In Silico prediction tools

Different *in silico* prediction tool sources were used to check the variant pathogenicity and effects. In table in Table 3.2. we listed these tools.

Table 3.2. *in silico prediction tools used in our study*

Prediction tool	**Source/ website**
ACMG: American College of Medical Genetics	https://www.acmg.net/
SIFT: Sorting Intolerant from Tolerant	https://sift.bii.a-star.edu.sg/
PolyPhen-2: Polymorphism Phenotyping v2	http://genetics.bwh.harvard.edu/pph2/
FATHMM: Functional Analysis through Hidden Markov Models	http://fathmm.biocompute.org.uk
M-CAP: Mendelian Clinically Applicable Pathogenicity	http://bejerano.stanford.edu/mcap
REVEL: Rare Exome Variant Ensemble Learner	https://sites.google.com/site/revelgenomics
CADD: Combined Annotation Dependent Depletion	https://cadd.gs.washington.edu/score
PROVEAN: Protein Variation Effect Analyzer	http://provean.jcvi.org/index.php
eVai pathogenicity score: Expert Variant Interpreter	https://www.engenome.com/evai/
Mutation Taster	https://www.mutationtaster.org/
Mutation Assessor	http://mutationassessor.org/r3/
MutPred2	http://mutpred.mutdb.org/
SpliceAI	https://spliceailookup.broadinstitute.org/

3.2.2.4 Phenotype and diseases related database

- **The Human Phenotype Ontology (HPO)**

The Human Phenotype Ontology (HPO) provides a standardized, controlled terminology (vocabulary) of phenotypic anomalies associated with +7000 human diseases. Thousands of researchers, informaticians, physicians and electronic health record systems use HPO all over the world. Currently, the HPO is being developed using medical literature, Orphanet, OMIM, and DECIPHER. Until now, HPO includes over 13,000 disease terms and over 156,000 annotations to hereditary disorders. Each HPO term has a unique ID number such as *HP:0001875 for neutropenia* (Köhler et al., 2019). Each term is also assigned to one of the five sub ontologies; phenotypic abnormality (the main subontology of the HPO and contains descriptions of clinical abnormalities), inheritance patterns (describe the mode of inheritance and contains terms such as autosomal recessive inheritance), onset/clinical course (outlines how a disease develops, progresses over time), modifiers of abnormalities (outline common clinical symptom modifiers, for instance, the rate at which the disease progresses, the triggering factors, the location, or the severity) and Frequency (Obligate, Frequent, and Occasional). Additionally, a panel of genes related to each HPO term are available (Köhler et al., 2021) (https://hpo.jax.org/app/).

- **Online Mendelian Inheritance in Man (OMIM).**

OMIM is a free, comprehensive, and reliable compendium of human genes and phenotype and their relationship. OMIM has links to a variety of related and complementary databases, making it simple to find more information on a certain topic. In January 2011, OMIM created a new official website, OMIM.org (http://omim.org). Since then, OMIM is updated daily and contains data on all known mendelian diseases and more than 16,000 genes. All phenotypes and genes are documented separately and given six-digit unique identifiers (MIM numbers). For instance, the code of Shwachamn-Diamond Syndrome is 260400, and 607444 is the code related to the SBDS gene (Amberger et al., 2015; Hamosh et al., 2021).

3.2.2.5 Varsome database

The website of varsome (www.varsome.com) is a search engine for human genetic variant analysis. It is a community-driven project designed to share global expertise in human variants. VarSome includes data from more than 70 external databases and now has more than 400,000 users of healthcare and life sciences professionals worldwide. Users can share evidence on variants, classify and link research publications and findings and create a unique resource in the genomics landscape. VarSome's database contains over 33 billion data points that describe over 500 million genetic variations. Users can search by gene name, transcript symbol, genomic location, variant ID or HGVS nomenclature.

Varsome uses an automatic variant classifier to evaluate the variant pathogenicity according to the ACMG guidelines, the most widely clinical guidelines used. Variants can be classified into pathogenic, likely pathogenic, likely benign, benign and VUS (Richards et al., 2015). Additionally, pathogenicity predictions from 20 different *in silico* prediction tools such as DANN, SIFT, PROVEAN, PolyPhen-2, CADD, MetaSVM, Mutation Taster, and FATHMM, are also included. The frequency of the alternate allele in different sub-populations is taken from gnomAD (Lek et al., 2016), ICGC somatic (Hudson et al., 2010), and Kaviar3 (Glusman et al., 2011). Clinically relevant data including inheritance mode, associated conditions, and publications are taken from Candida Genome Database (CGD) (Solomon et al., 2013). Variants are also connected to any related phenotypes according to the HPO (Kopanos et al., 2019).

3.2.2.6 Protein alignment and BLAST using UniProt

UniProt, or the Universal Protein Resource, provides an up-to-date, comprehensive body of protein information at a single site (UniProt Consortium, 2015). To build upon this protein data and to aid analysis, UniProt provides three main tools: 'BLAST' (Basic Local Alignment Search Tool), 'Align' multiple sequence alignment tools, and 'Retrieve/ID Mapping' for batch retrievals of UniProt entries and ID mapping between UniProt and external databases. We used UniProt to perform alignment to check the conservation for different amino acid changes.

3.3 Sanger sequencing validation

Variants of interest were validated using Sanger sequencing. Primer3web (https://primer3.ut.ee/) was used to design the primers. PCRs reactions were performed in 25µL of reaction volume containing 50 ng of genomic DNA, 0.5 µM of primers, 2 µl of dNTPs Mixture (2.5 mm each), 2.5 µl of 5X PrimeSTAR GXL Buffer, and 1.25U of PrimeSTAR GXL DNA Polymerase (TAKARA BIO INC.). PCR products were evaluated using

2% agarose gel electrophoresis and cleaned up with a PCR Clean-up NucleoSpin® kit (Macherey-Nagel). Sequencing reactions were performed using BrilliantDye Terminator Cycle sequencing kit version 3.1 (Nimagen). The final reaction volume was 10 µL containing: 1.5 µL of purified PCR, 1.5 µL of 5µM forward or reverse primer, 4.5 µL of DEPC water, 2 µL of 5X Buffer and 0.5 µL of BrilliantDye Terminator (kept always covered). The sequencing reaction conditions were: 1. 96°C 1', 2. 96°C 10'', 3. 50°C 5'', 4. 60°C 4', 5. go to 2. for 24 cycles, 6. 4°C pause.

Post-Sequencing Reaction Purification was applied using DyeEx 2.0 Spin Kit Qiagen and for each sample the 2 µL of the purified eluate was added to 10 µL of formamide, transferring the final volume to a 96-well plate. After 5' of denaturation and 3' at -18°C, the plate with the samples was inserted into the available sequencer (3500 DX Genetic Analyzer). The files obtained were analyzed by Finch TV software.

3.4 Variant filtering used in our study

We used eVai platform to filter the VCF for 16 SDS patient in order to achieve the objectives of the following sub-studies (Nicora et al., 2022):

3.4.1: Identification of variants in other genes related to SDS or SDS-like phenotypes:

We filtered out all VCF files using **Filter 1** searching only for coding variants with good quality (QUAL ≥ 30) in *DNAJC21*, *EFL1, SRP54* and *EIF6* genes.

3.4.2: Distribution of germline variants related to the development of hematological abnormalities in 16 patients affected with SDS:

All variants were filtered according to the following criteria:

1. **Filter 2:** variants in genes related to the HPO terms of **MDS and/or AML.**
    - **Filter 2.1:** Variants related to the HPO term describing MDS and/ or AML with good quality (QUAL ≥ 30), and pathogenicity score ≥ 0 were selected.
    - **Filter 2.2**: Variants with good quality (QUAL ≥ 30), and MAF ≤ 0.05 were included. Intronic variants were excluded.
2. **Filter 3:** Variants in genes related to HPO of **neutropenia** and good quality (QUAL ≥ 30) were selected. Variants with low or no impact on gene function (pathogenicity Score < 0), and deep intronic variants were excluded.
3. **Filter 4:** Variants in genes related to HPO of **bone marrow hypocellularity** and good quality (QUAL ≥ 30) were selected. Variants with low or no impact on gene function (pathogenicity Score < 0), and deep intronic variants were excluded.

4. **Filter 5:** Variants in genes related to HPO of **thrombocytopenia** and good quality (QUAL ≥ 30) were selected. Variants with low or no impact on gene function (pathogenicity Score < 0), and deep intronic variants were excluded.

3.4.3: Phenotypic variation in two siblings affected with SDS: the use eVai suggests clinical relevance of a variant in the KMT2A gene:

Two different types of filters were applied:

- **Filter 6:** Variants with good quality (QUAL ≥ 30) and high pathogenicity score (pathogenicity score ≥ 3) were selected for UPN 42 and UPN 43; then, shared variants were excluded.
- **Filter 7:** Variants with good quality (QUAL ≥ 30) and that were related to each HPO term described for UPN 42 and UPN 43 in table 3.3 were selected. Variants with low or no impact on gene function (pathogenicity Score < 0), and deep intronic variants were excluded.

Table 3.3. *HPO terms that describe UPN42 and UPN43 phenotypes*

UPN42	UPN43
HP:0001875 Neutropenia	HP:0000085 Horseshoe kidney
HP:0005528 Bone marrow hypocellularity	HP:0001875 Neutropenia
HP:0001738 Exocrine pancreatic insufficiency	HP:0005528 Bone marrow hypocellularity
HP:0001873 Thrombocytopenia	HP:0001738 Exocrine pancreatic insufficiency
HP:0040088 Abnormal lymphocyte count	HP:0003025 Metaphyseal irregularity
HP:0012189 Hodgkin lymphoma	HP:0012758 Neurodevelopmental delay
	HP:0001873 Thrombocytopenia
	HP:0000028 Cryptorchidism
	HP:0002719 Recurrent infection
	HP:0000431 Wide nasal bridge
	HP:0000316 Hypertelorism
	HP:0002474 Expressive language delay

3.5 Variant interpretation techniques used in our study

We evaluated all variants resulting from filters 1, 2, 3, 5, 5, 6 and 7 according to their chromosomal position, frequency according to gnomAD, mode of inheritance, mutation consequencing, literature using Pubmed and google scholar databases, related conditions using OMIM MalaCard and HPO databases, bioinformatic prediction tools including eVai pathogenicity score, ACMG, SIFT, MVP, FATHMM, Mutation Taster, M-CAP, and CADD were evaluated for some variants. The ACMG classification was checked using Versome,

franklin and eVai. The database of human biological pathway unification (PathCards) was used to identify the pathway for the gene of interest.

3.6 Cytogenetics

Repeated conventional and molecular cytogenetic analyses were performed on bone marrow yearly since 2002, almost regularly, up to 2021. The methods used included routine cytogenetics, fluorescent in Situ Hybridization (FISH) on metaphases and nuclei with informative probes and libraries, and array-based Comparative Genomic Hybridization (a-CGH), according to the methods reported in Pressato et al., 2015 (Pressato et al., 2015). (The cytogenetic work was performed in collaboration with Dr. Roberto Valli and Prof. Francesco Pasquali from University of Insubria, Varese, Italy).

3.7 Structural analysis

For structural visualization and evaluation of the effect of the Phe34Leu mutation, the model of eIF6 was extracted from PDB code 6LSS (pre-60 ribosome). This model had originally been generated by SWISS-MODEL (Waterhouse et al., 2018). and docked into the electron density of human pre-60S ribosomal particles by rigid-body fitting, followed by extensive manual rebuilding in COOT with a final local resolution comprised between 3.1 and 4.8 Å (Liang et al., 2020). (The structural analysis work has been done by Dr. Claudia Scotti, Department of Molecular Medicine, University of Pavia).

3.8 Statistical analysis

Statistical analysis was performed using IBM Statistical Product and Service Solutions (SPSS V.25). For quantitative variables including neutrophils and platelet counts, we calculated the mean, standard deviation (SD), and range. Pearson correlation (r) was used to determine the relationship between the number of variants and neutrophils and platelets count.

CHAPTER 4 – PATIENTS RECRUITMENT AND CHARACTERISTICS

4.1 Patients recruitment

In this study, a total of 16 Italian SDS patients were included. Patients have been referred from several national centers. All patients have been proven to carry biallelic pathogenic variants in the *SBDS* gene. Each patient was given a Unique Patient Number (UPN). The patients are included in the Italian registry for SDS disease (http://www.registroitalianosds.org/).

4.2 Data collection

All personal and medical information was requested from the Italian Shwachman Diamond Syndrome Registry (RI-SDS) (August 2022). Since 2011, the Azienda Ospedaliera Universitaria Integrata of Verona's Ethics Committee has accepted the RI-SDS as an observational study. Patients, their parents, or legal guardians provided informed consent for the collection of clinical, hematologic, and genetic data (Cesaro et al., 2020). Each participating center's investigator collected data using a written case report form that was filled out at the time of the genetic diagnosis and yearly after that. Clinical and hematologic evaluations every 6-12 months and 1-3 years, respectively, or more frequently in case of rapid and persistent cytopenia or symptoms of clonal development, were advised following the genetic diagnosis of SDS (Dror et al., 2011). Data for hemoglobin, mean corpuscular volume, absolute neutrophil count (ANC), platelet count, and white blood cell count were evaluated. An ANC of less than 1500 cells/ mcL, a platelet count of less than 150,000 mcL, and a hemoglobin level of less than 10 g/dL were used to characterize neutropenia, thrombocytopenia, and anemia, respectively. Bone marrow smears were examined by expert pediatric hematologists and the diagnoses of MDS and AML were made based on the blast count found in peripheral blood and bone marrow following the 2008 World Health Organization standards. For other hematological data, we relied on physicians' reports when available (Cesaro et al., 2020).

4.3 Genetic characteristics

All 16 patients have been confirmed to carry biallelic pathogenic variants in the *SBDS* gene using the Sanger sequencing method described in chapter 3, section 3.3. The pathogenic variant (258+2T>C) was found in all patients (N=16, 100%) as the 1st allele. Eight patients (50%) are harbouring the nonsense variant (183_184TA>CT) in the 2nd allele. Figure 4.1 illustrates the percentage of each different *SBDS* mutation in the 2nd allele, considering that the 1st allele is always mutated with 258+2T>C mutation, while Table 4.1 shows the patient's UPNs and their *SBDS* pathogenic variants.

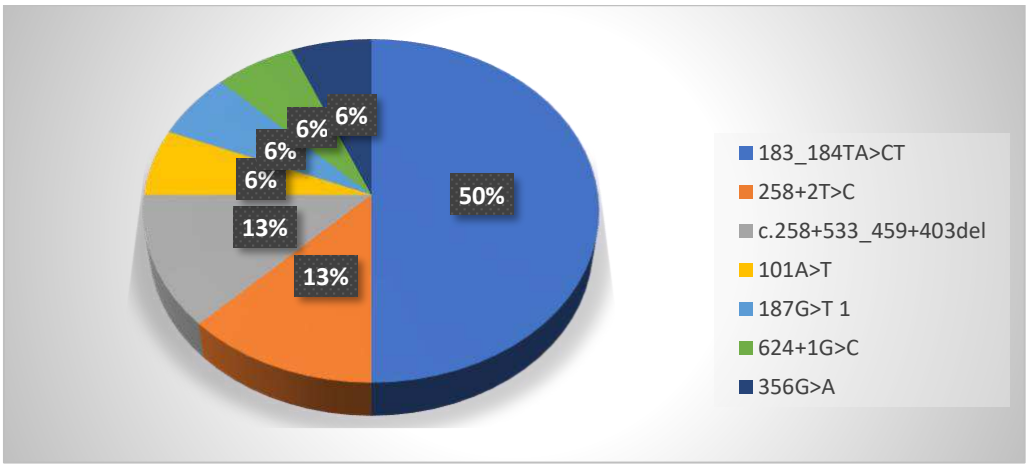


***Figure 4.1**: Graphical representation of genotypes found in 16 SDS patients. The pie chart describes the percentage of each different SBDS mutation in one allele, considering that the other allele is always mutated with 258+2T>C mutation.*

***Table 4.1**. The patient's UPNs and their SBDS pathogenic variants.*

n. case	ID case	*SBDS* mutations
1	UPN 20	258+2T>C/183_184TA>CT
2	UPN 6	258+2T>C/101A>T
3	UPN 68	258+2T>C/183_184TA>CT
4	UPN 57	258+2T>C/187G>T 1
5	UPN 58	258+2T>C/183_184TA>CT
6	UPN 15	258+2T>C/183_184TA>CT
7	UPN 65	258+2T>C/258+2T>C
8	UPN 64	258+2T>C/624+1G>C

9	UPN 42	258+2T>C/c.258+533_459+403del
10	UPN 43	258+2T>C/c.258+533_459+403del
12	UPN 2	258+2T>C/356G>A
13	UPN 62	258+2T>C/183_184TA>CT
14	UPN 85	258+2T>C/183_184TA>CT
15	UPN 24	258+2T>C/183_184TA>CT
16	UPN 51	258+2T>C/258+2T>C
17	UPN 45	258+2T>C/183_184TA>CT

4.4 Clinical characteristics

4.4.1 Human Phenotype Ontology (HPO) terms identification

Based on clinical information available for all patients, the Human Phenotype Ontology (HPO) database (https://hpo.jax.org/app/) was used to select the HPO terms and codes describing the patient's phenotypes (Table 4.2). In table 4.2, we also added the patient's sex and dates of birth (DoB).

Table 4.2. *HPO terms that describe the patients' phenotypes and their sex and dates of birth.*

Pt No.	UPN	Sex	DoB	HPO terms and codes
1	UPN 20	Male	1973	*Failure to thrive (HP:0001508)* *Growth delay (HP:0001510)* *Short stature (HP:0004322)* *Feeding difficulties (HP:0011968)* *Bone marrow hypocellularity (HP:0005528)* *Neutropenia (HP:0001875)* *Thrombocytopenia (HP:0001873)* *Metaphyseal irregularity (HP:0003025)* *Abnormal clavicle morphology (HP:0000889)* *Genu varum (HP:0002970)* *Low femoral bone density (HP:0031163)* *Osteoporosis (HP:0000939)* *Metaphyseal sclerosis (HP:0004979)* *Lumbar scoliosis (HP:0004626)*
2	UPN 06	Male	1992	*Growth delay (HP:0001510)* *Short stature (HP:0004322)* *Bone marrow hypocellularity (HP:0005528)* *Seizure (HP:0001250)*
3	UPN 68	Male	2000	*Failure to thrive (HP:0001508)* *Bone marrow hypocellularity (HP:0005528)* *Thrombocytopenia (HP:0001873)* *Osteopenia (HP:0000938)* *Fibrous dysplasia of the bones (HP:0010734)* *Cognitive impairment (HP:0100543)* *Recurrent infections (HP:0002719)*
4	UPN 57	Female	1982	*Failure to thrive (HP:0001508)* *Cognitive impairment (HP:0100543)* *Exocrine pancreatic insufficiency (HP:0001873)* *Bone marrow hypocellularity (HP:0005528)* *Neutropenia (HP:0001875)* *Thrombocytopenia (HP:0001873)*

				Persistence of hemoglobin F (HP:0011904)
				Abnormality of blood-forming (HP:0001871)
				Pelvic bone asymmetry (HP:0010453)
				Spinal malformation (HP:0002390)
5	UPN 58	Male	2001	*Growth delay (HP:0001510)*
				Neutropenia (HP:0001875)
				Exocrine pancreatic insufficiency (HP:0001873)
				Recurrent infections (HP:0002719)
				Incoordination (HP:0002311)
				speech delay (HP:0000750)
				Psychomotor delay (HP:0025356)
6	UPN 15	Female	1994	*Bone marrow hypocellularity (HP:0005528)*
				Neutropenia (HP:0001875)
				Recurrent infections (HP:0002719)
				Lower limb asymmetry (HP:0100559)
				Abnormal foot morphology (HP:0001760)
7	UPN 65	Male	2001	*Failure to thrive (HP:0001508)*
				Bone marrow hypocellularity (HP:0005528)
				Neutropenia (HP:0001875)
				Thrombocytopenia (HP:0001873)
				Recurrent infections (HP:0002719)
				Spinal malformation (HP:0002390)
				Abnormality of the lower limb (HP:0002814)
				Perthes disease (HP:0005743)
				Cognitive impairment (HP:0100543)
8	UPN 64	Male	1991	*Exocrine pancreatic insufficiency (HP:0001873)*
				Bone marrow hypocellularity (HP:0005528)
				Neutropenia (HP:0001875)
				Abnormality of blood-forming (HP:0001871)
9	UPN 42	Female	1987	*Hodgkin lymphoma (HP:0012189)*
				Bone marrow hypocellularity (HP:0005528)
				Neutropenia (HP:0001875)
				Thrombocytopenia (HP:0001873)
				Abnormal lymphocyte count (HP:0040088)
				Exocrine pancreatic insufficiency (HP:0001873)
10	UPN 43	Male	1994	*Exocrine pancreatic insufficiency (HP:0001873)*

				Neutropenia (HP:0001875)
				Thrombocytopenia (HP:0001873)
				Bone marrow hypocellularity (HP:0005528)
				Neurodevelopmental delay (HP:0012758)
				Expressive language delay (HP:0002474)
				Horseshoe kidney (HP:0000085)
				Cryptorchidism (HP:0000028)
				Metaphyseal irregularity (HP:0003025)
				Recurrent infections (HP:0002719)
				Wide nasal bridge (HP:0000431)
				Hypertelorism (HP:0000316)
12	UPN 02	Male	1999	*Neutropenia (HP:0001875)*
				Exocrine pancreatic insufficiency (HP:0001873)
				Thrombocytopenia (HP:0001873)
				Bone marrow hypocellularity (HP:0005528)
13	UPN 62	Female	2005	*Failure to thrive (HP:0001508)*
				Neutropenia (HP:0001875)
				Exocrine pancreatic insufficiency (HP:0001873)
				Thrombocytopenia (HP:0001873)
				Bone marrow hypocellularity (HP:0005528)
				Mandibular prognathia (HP:0000303)
				Spinal malformation (HP:0002390)
14	UPN 85	Male	*1995*	*Decreased circulating antibody level (HP:0004313)*
				Thrombocytopenia (HP:0001873)
				Leukopenia (HP:0001882)
				Neutropenia (HP:0001875)
				Scoliosis (HP:0002650)
				Bone marrow hypocellularity (HP:0005528)
				Recurrent infections (HP:0002719)
				Exocrine pancreatic insufficiency (HP:0001738)
				Asthma (HP:0002099)
				Accessory spleen (HP:0001747)
				Mitral regurgitation (HP:0001653)
15	UPN 24	female	1989	*Neutropenia (HP:0001875)*
				Exocrine pancreatic insufficiency (HP:0001873)
				Thrombocytopenia (HP:0001873)

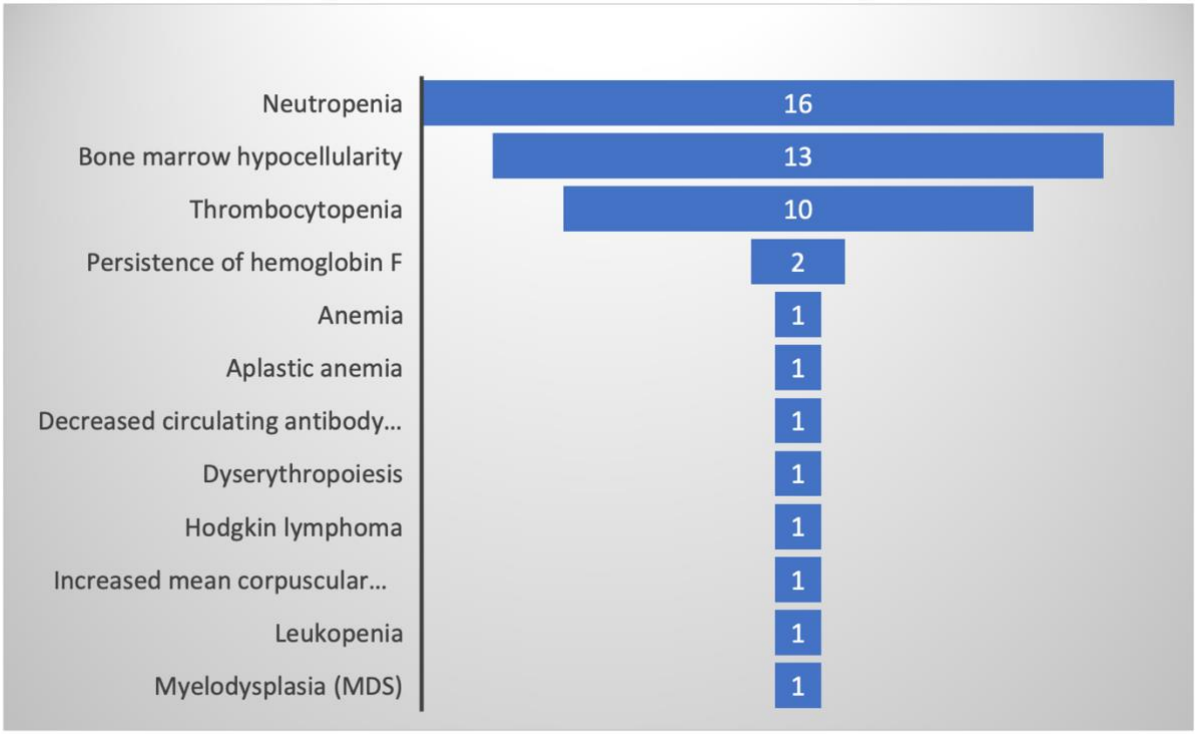
				Anemia (HP:0001903)
				Hepatopathy (HP:0001410)
				Dyserythropoiesis (HP:0001877)
				Increased mean corpuscular volume (MCV) (HP:0005518)
16	UPN 51	Female	2000	*Bone marrow hypocellularity (HP:0005528)*
				Exocrine pancreatic insufficiency (HP:0001873)
				Short stature (HP:0004322)
				Elevated hepatic transaminase (HP:0002910)
				learning disability (HP:0001328)
				Carious teeth (HP:0000670)
				Psychomotor delay (HP:0025356)
17	UPN 45	Male	2005	*Exocrine pancreatic insufficiency (HP:0001873)*
				Diabetes mellitus (HP:0000819)
				Myelodysplasia (MDS) (HP:0002863)
				Persistence of hemoglobin F (HP:0011904)
				Short stature (HP:0004322)
				Elevated hepatic transaminase (HP:0002910)
				Neutropenia (HP:0001875)
				Aplastic anemia (HP:0001915)
				History of bone marrow transplant (HP:0032557)
				Systolic heart murmur (HP:0031664)
				Carious teeth (HP:0000670)
				Tics (HP:0100033)
				Intellectual disability, mild (HP:0001256)

4.4.2 Clinical description

- **Hematological manifestations:**

As previously introduced, neutropenia is essential for the disease diagnosis and it was found in all patients (N=16,100%). Bone marrow hypocellularity was found in most patients (N=13,81.25%). Thrombocytopenia and persistence of hemoglobin F were reported in 10 (62.5%) and 2 (12.5%) patients respectively. Additional features that have been reported include anemia decreased circulating antibody level, dyserythropoietic aplastic anemia, Hodgkin lymphoma, increased mean corpuscular volume (MCV), Leukopenia, and Myelodysplasia (MDS) (Figure 4.2).

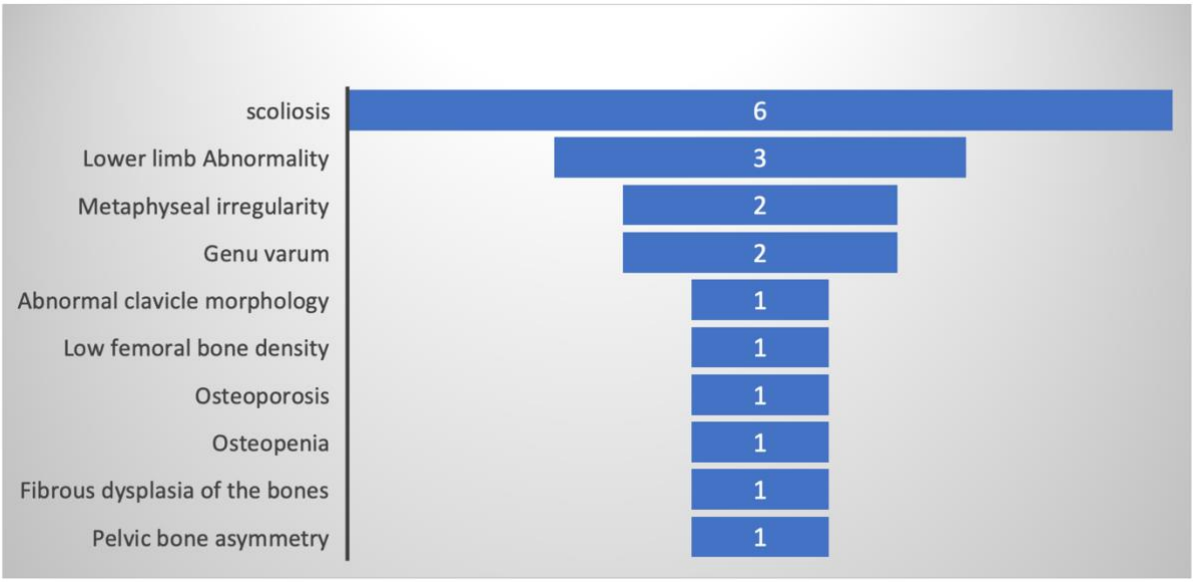
Figure 4.2. *Distribution of hematological features among 16 SDS patients.*



- **Skeletal abnormalities:**

The skeletal abnormalities were varied and found in 7 patients. Signs of scoliosis were reported in 6 cases. Metaphyseal irregularity and lower limb abnormalities were found in 2 and 3 patients respectively. Features including genu varum, abnormal clavicle morphology, low femoral bone density, osteoporosis, osteopenia, fibrous dysplasia of the bones, and pelvic asymmetry have been reported in some patients (Figure 4.3).

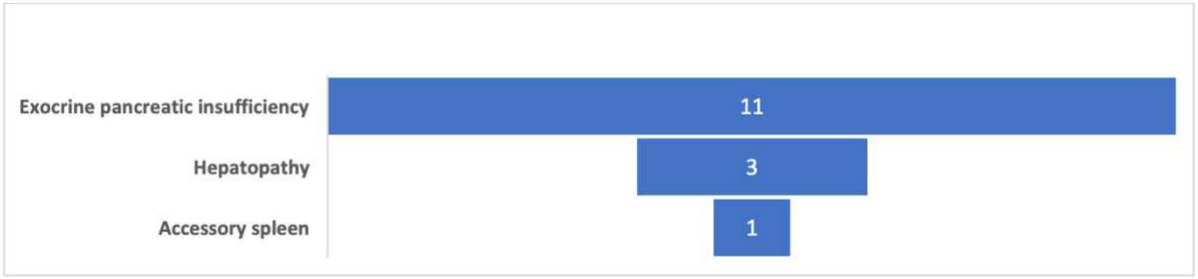
Figure 4.3. *Skeletal abnormalities found in 16 SDS patients.*



- **Gastrointestinal manifestations**

Out of 16 patients, 11 (68.75%) of them have been diagnosed with Pancreatic insufficiency. Two additional features including hepatopathy and accessory spleen have been reported in 3 and 1 patient respectively (Figure 4.4).

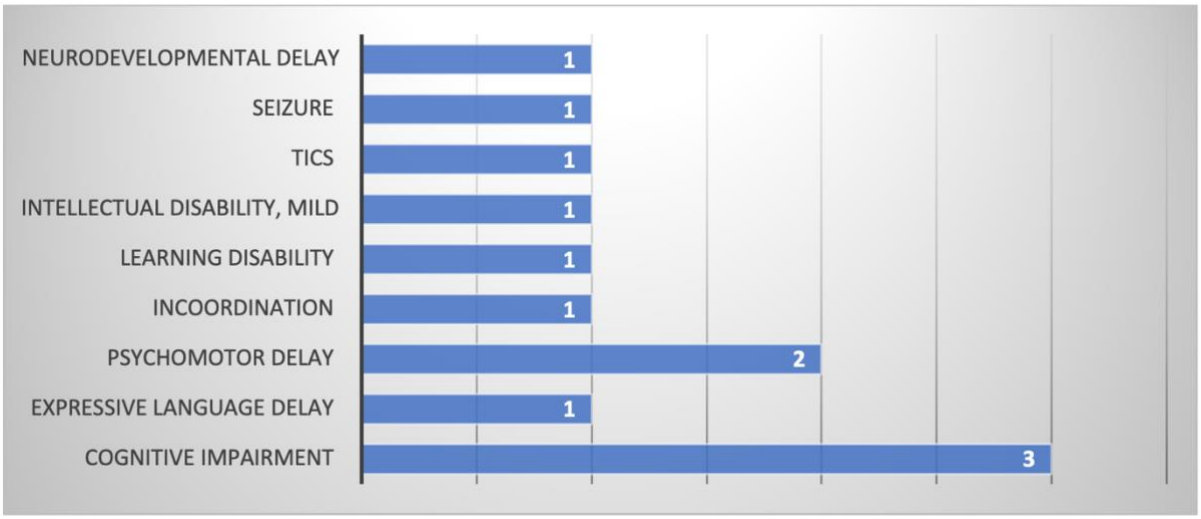
Figure 4.4. *Distribution of pancreatic and gastrointestinal features*



- **Neuropsychological issues**

Neuropsychological issues have been reported in 50% of patients. Nine Neuropsychological issues have been reported. Three patients have been diagnosed with cognitive impairment. Two subjects had expressive language and speech delays. Two patients also had a psychomotor delay. One patient at least has incoordination, learning disability, intellectual disability, tics, seizure and neurodevelopmental delay (Figure 4.5).

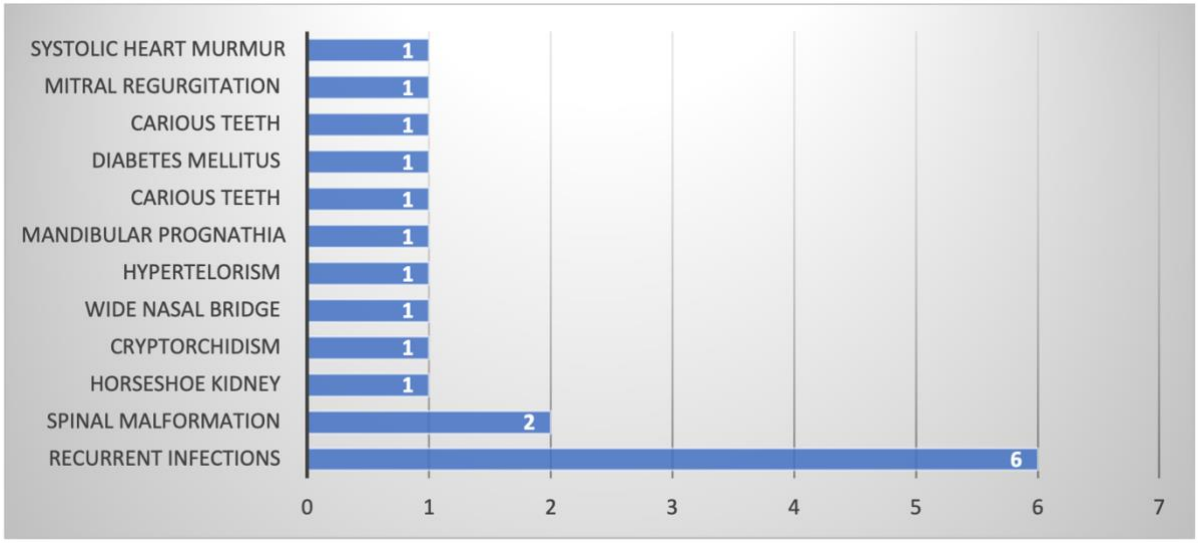
Figure 4.5. *Distribution of neuropsychological issues*



- **Other manifestations**

Other features have been found, including those of the immune, cardiac, renal, dental, and endocrine systems. Recurrent infection and spinal arteriovenous malformation have been found in 6 and 2 patients respectively. Cardiac manifestations including mitral regurgitation and systolic heart murmur were found in 2 patients. dental problems such as carious teeth were reported in 2 subjects. Facial dysmorphisms of mandibular prognathia, wide nasal bridge and hypertelorism have been found in some patients (Figure 4.6).

Figure 4.6. *Other manifestations found in our cohort*



CHAPTER 5 – RESULTS

The result chapter is divided into 3 sub-studies as following:

5.1 Identification of variants in other genes related to SDS or SDS-like phenotypes

The major part of this study was published in Frontiers in Genetics Journal on 22 of August 2022 (https://doi.org/10.3389/fgene.2022.896749). The full version of the paper is attached in the appendix section of the thesis.

In this study, using the new Expert Variant Interpreter (eVai), we searched in our patients, for other variants in genes related to SDS or SDS-phenotype including *DNAJC21, EFL1 SRP54* and *EIF6*.

5.1.1 finding of variants in other genes related to SDS or SDS-like phenotypes

While reviewing our WES files, we identified 8 variants inherited in heterozygous status in the *DNAJC21, EFL1,* and *EIF6* genes. No variants in the *SRP54* were found. (Table 5.1).

Table 5.1. *Information about the variants in the DNAJC21, EFL1 and EIF6 found in our cohort*

***DNAJC21* variants**

UPNs	HGVS_Coding	HGVS_Protein	Zyg	MAF	*In silico prediction tools*						
					eVai	SIFT	MT	MA	M-CAP	PROVEAN	ACMG
57	c.1024G>A	p.Val342Met	het	0.0030	1.5	B	D	B	B	B	B
58	c.1134A>G	p.Pro378Pro	het	0.050	1	B	B	B	D	B	B
58 and 2	c.1407C>G	p.Val469Val	het	0.050	0	B	B	B	B	B	B
64	c.826G>A	p.Glu276Lys	het	0.0001	2	D	D	B	B	B	VUS

***EFL1* variants**

UPNs	HGVS_Coding	HGVS_Protein	Zyg	MAF	*In silico prediction tools*						
					eVai	SIFT	MT	MA	M-CAP	PROVEAN	ACMG
6, 15, 57 and 62	c.2132A>G	p.Lys711Arg	het	0.130	1	B	D	B	B	B	B
6, 15, 57 and 62	c.1849A>G	p.Ile617Val	het	0.140	0.5	B	B	B	B	B	B
86	c.1486C>T	p.Leu496Phe	het	0.010	0.5	B	B	B	B	B	B

***EIF6* variant**

UPNs	HGVS_Coding	HGVS_Protein	Zyg	MAF	*In silico prediction tools*						
					eVai	SIFT	MT	MA	M-CAP	PROVEAN	ACMG
2	c.100T>C	p.Phe34Leu	Het	0.0002	5.5	D	D	D	D	D	VUS

Zyg: zygosity, **MAF**: minor allele frequency, **SIFT**: Sorting Intolerant from Tolerant, **MT**: Mutationt Taster, **MA**: Mutation Assessor, **ACMG**: American College of Medical Genetics guidelines, **B**: benign, **LB**: Likely Benign, **P**: Pathogenic, **LP**: Likey pathogenic, **D**: Damaging

Among all variants, only the variant (c.826G>A) in the *DNAJC21,* found in UPN64, and the variant (c.100T>C) in the *EIF6* gene, found in UPN2, were classified as VUS according to the ACMG guidelines. Note that, the variant c.826G>A in the *DNAJC21* and *EFL1* variants (c.2132A>G, c.1849A>G and c.1486C>T) were described in a previous publication for our research group (Morini et al., 2019).

Since the variants in the *DNAJC21* and *EFL1* have been discussed before, our focus in this chapter will be on the variant (c.100T>C; p. Phe34Leu) in the *EIF6* gene found in UPN2.

5.1.2 UPN2 Case Report

Our patient, a 23-year-old male (born in1998), identified as UPN 2 (Unique Patient Number) in previous work by our group (Valli et al., 2019), is the second child of healthy non-consanguineous Italian parents. UPN 2 was diagnosed as affected by SDS in 2001 based on the presence of neutropenia and pancreatic insufficiency.

Since diagnosis, an annual follow up has been performed at the Paediatric Onco-hematology unit. Over the last ten years, the patient has maintained a stable haematological picture.

At the latest clinical evaluation (2021), his height was 155,0 cm, his weight 50,0 kg, and his chest circumference 77.5 cm, all at 25-30 centiles of height and weight distribution of SDS male/female patients (personal communication by M. Cipolli and G. Tridello). At the same time, no pathological changes were observed in both lower limbs, and normal bone density was reported. Similarly, no structural changes of thoracic cage were present. His school performance was low average. At the same clinical evaluation of 2021, haemoglobin and RBC counts were normal (14.9, normal value, nv: 13.2-17.3 g/dL and 4.35x10^6 per µl, nv 4.30-5.70x10^6 per µl, respectively). Neutrophil's count was low (0.9x10^3, nv: 2.0-8.0x10^3 per µl, and ranging between 0.5 and 0.7x10^3 per µl in previous tests), lymphocytes count was normal (1.8x10^3µl, nv: 1.5-4.0x10^3 per µl) and platelets were slightly reduced (120x10^3 per µl, nv: 150-400x10^3 per µl, and ranging between 100 and 130 x10^3 per µl in previous tests). Bone marrow biopsy confirmed, as the previous controls, a stable cellularity (20%), and immunostaining with anti-p53 antibody was consistently negative. No evidence of any change suggesting myelodysplasia was reported.

Routine biochemical tests were within normal limits; serum amylase and pancreatic isoamylase were low, 14.0 mU/ml and 5.0 mU/ml respectively (nv: 25.0-125.0 mU/ml and 8.0-53.0 mU/ml, respectively). Serum lipase was within normal limits: 36.0 mU/ml (nv: 8.0 - 58.0 mU/ml). Creon tablets as a treatment for the exocrine pancreatic

insufficiency were prescribed. S-Parathyroid hormone (PTH) levels were slightly elevated during the last two checks, corresponding to 112.3 and 84.5 pg/ml values (nv: 12,0 - 72,0 pg/ml). Vitamin D, calcium and potassium were at the normal levels. The patient had received his third dose of COVID-19 vaccine without any major complication.

Similarly, a recent survey carried out by an American SDS Registry, confirmed no worrying clinical effect in three SDS patients who have been vaccinated against COVID-19 (Galletta et al., 2022).

In the years 2008-2010 bone marrow karyotype analysis detected a small clone with the i(7)(q10), but later (years from 2011 to 2020) it disappeared. Furthermore, no structural abnormality of chromosome 20 was ever identified (***Dr. Valli and Prof. Pasquali***).

5.1.3 Molecular and bioinformatics analysis for UPN2

The clinical diagnosis of SDS was confirmed by the presence of one of the common mutations [c.258+2T>C, p.Cys84fs3, paternal] and a second variant [c.356G>A, p.Cys119Tyr, maternal] in *SBDS*. The latter is included by Finch et al. among disease associated missense variants, since, on the basis of functional studies, it was evaluated as a mutation affecting SBDS protein stability (Finch et al., 2011).

The variant (c.100T>C; p. Phe34Leu) in the *EIF6* was confirmed using Sanger sequencing for the patient and assessed its maternal origin (Figure 5.1).

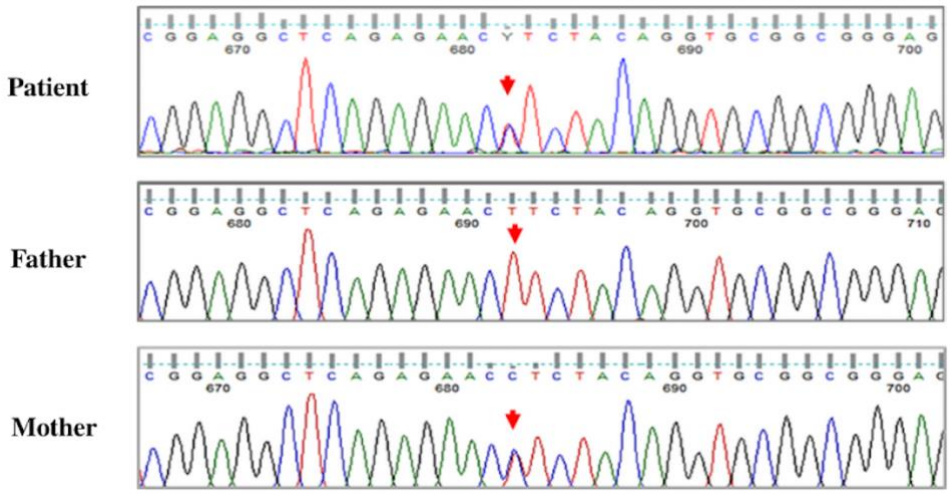


Figure 5.1*. Part of electropherogram from the proband and their parents, showing the substitution T>C in the heterozygous status at the position c.100 maternal inherited*

According to Genome Aggregation Database (gnomAD), the variant is a rare one as its frequency is 0.0002002. These results were obtained in DNA from a blood sample, but we checked the variant in DNAs also from eight bone marrow specimens obtained at regular follow-ups (the latest one in 2021): all of these showed the stable presence of the heterozygous variant, with a ratio 1:1 to normal allele.

Protein sequence alignment using Uniprot (www.uniprot.org) showed that the amino acid Phenylalanine in position 34 is highly conserved among different species such as Human, Mouse, Dog, Elephant, Monkey, Zebrafish, Frog, Reptiles, and S. cerevisiae (Figure 5.2).

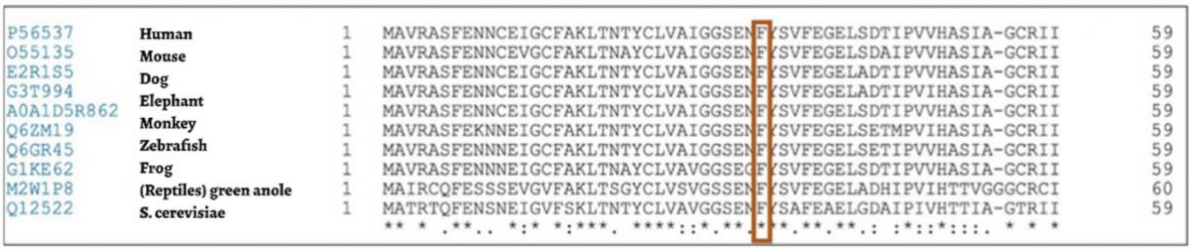


Figure 5.2. *Protein sequence alignment using UniProt shows absolute conservation of F34 throughout different species (human, mouse, dog, elephant, monkey, zebrafish, frog, reptiles, and S. cerevisiae.*

We then filtered the WES results for the presence of variants in genes related to the development of MDS or leukemia as reported by several authors (Lindsley, 2017; Steensma, 2017; Bluteau et al., 2018; Obrochta and Godley, 2018; Armstrong et al., 2018; Kennedy et al., 2021). The variants with a frequency ≤ 0.01 were validated by Sanger sequencing in the patient and his parents. These variants were entered in Table 5.2 with their HGVS_coding, zygosity, MAF, pathogenicity prediction according with various specific tools, and parental origin.

Table 5.2. Variants found in UPN 2 with a frequency ≤0.01 in genes related to MDS and AML.

Gene	HGVS_Coding	Parental origin	Zyg	MAF	*In silico prediction tools*						
					eVai	SIFT	MT	MA	M-CAP	PROVEAN	ACMG
ASXL1	c.3631G>A	Father	het	0.0	1.5	B	D	B	B	B	VUS
JAK2	c.44C>T	Father	het	0.0000159	1.5	D	B	B	D	D	VUS
GNAS	c.2149G>T	Father	het	0.0	0	N/A	B	N/A	D	D	P

Zyg: zygosity, **MAF**: minor allele frequency, **SIFT**: Sorting Intolerant from Tolerant, **MT**: Mutationt Taster, **MA**: Mutation Assessor, **ACMG**: American College of Medical Genetics guidelines, **B**: benign, **P**: Pathogenic, **D**: Damaging

5.1.4 Structural analysis

The results of the structural analysis for the F34L variant and described below highlight the evidence of a large decrease of protein stability. We can therefore envisage that the F34L variant could impair eIF6 folding, with a reduction of binding to the nascent 60S ribosomal subunit through protein L23 and a possible consequent reduction of binding of protein GTPBP4, which is also involved in ribosomal biogenesis.

The protein eIF6 consists of five *quasi*-identical alpha/beta subdomains arrayed about a five-fold axis of pseudosymmetry (Figure 5.3, A). Residue F34 (blue sticks) is the core part of a chain of hydrophobic interactions mediated by a cluster of five aromatic residues (F7, F16, F34, Y35 and F38) (pink sticks) belonging to the N-terminal domain and one, F205, belonging to the C-terminal domain (Figure 5.3, B). This residue arrangement, involving helix 2A and β -strand 2E, where the group of three residues: F34, Y35, F38, and F205 are located, respectively (nomenclature according to Groft et. al,2000 (Groft et al., 2000, p. 6), contributes to stabilize the "velcro" strategy adopted in the pentein fold of eIF6 (Fülöp and Jones, 1999), where β -strand 2E at the C-terminus hydrogen bonds with a β-strand from the N-terminal subdomain. The flat protein surfaces thus formed are necessary for interaction with ribosomal protein L23, a component of the 60S subunit, on one side, and with GTPBP4 (Nucleolar GTP-binding protein 1), also involved in the 60S biogenesis, on the other (Figure 5.3, C and D). The F34L variant lacks the central phenylalanine of the cluster, interrupts this regular pattern and introduces clashes, represented by solid red disks in Figure 5.3 E and F, where both leucine rotamers are considered. Despite the similar hydrophobic nature of the two residues, the reduction of the side chain size and the interruption of the aromatic stack explains why analysis by I-Mutant predicts a Gibbs free energy difference (ΔΔG) between the mutant and the wild-type of -0.87 Kcal/mole ***(Dr. Claudia Scotti).***

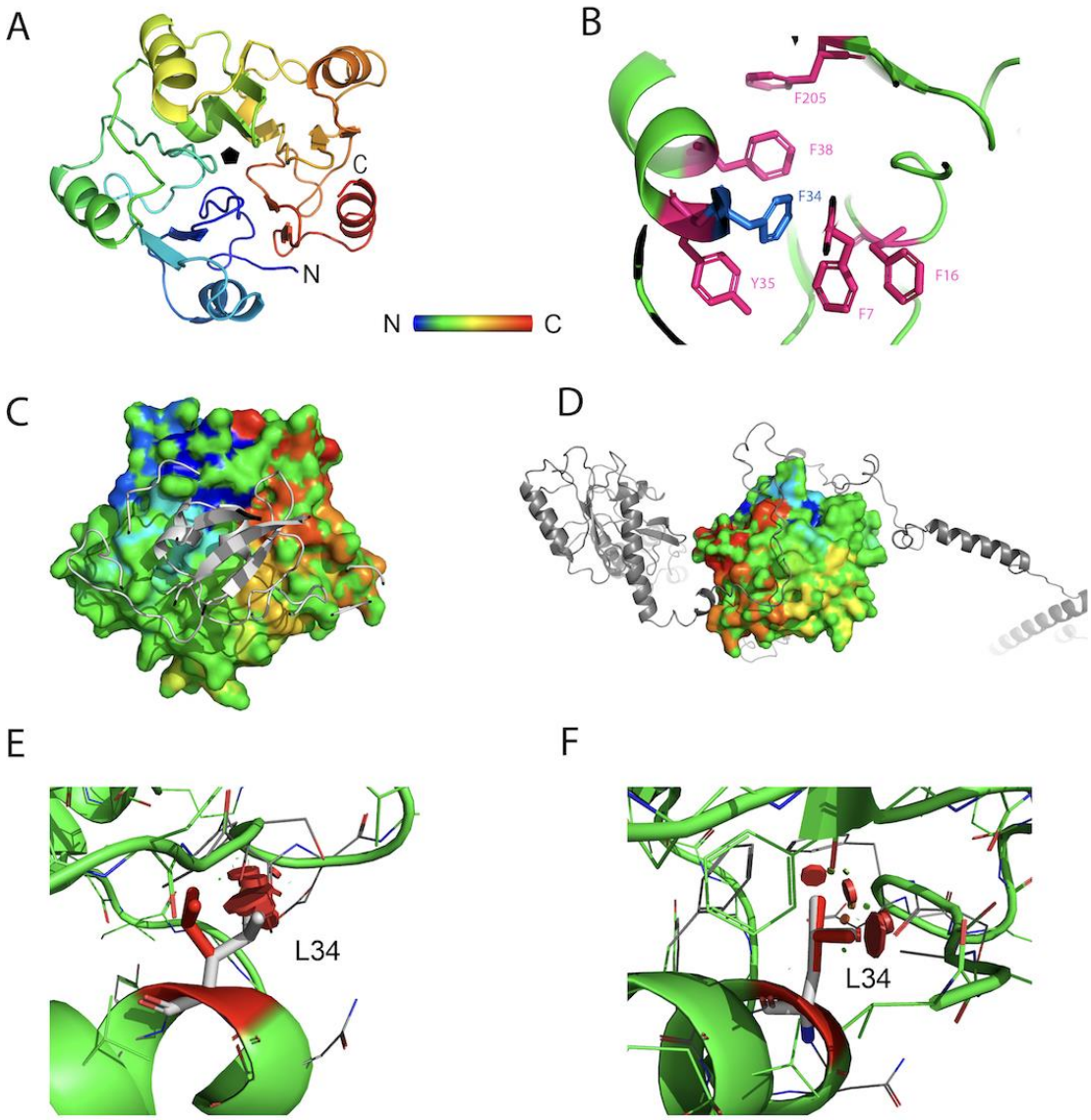


Figure 5.3. *Structural analysis of eIF6. (A) Rainbow-colored (blue: N-terminus, red: C-terminus) ribbon representation of eIF6, consisting of five quasi-identical alpha/beta subdomains arrayed about a five-fold axis of pseudosymmetry. (B) Residue F34 (blue sticks) is the core part of a chain of hydrophobic interactions mediated by a cluster of five aromatic residues (F7, F16, F34, F38, and Y35) belonging to the N-terminal domain and one, F205, belonging to the C-terminal domain (pink sticks). (C) Surface representation of eIF6 interacting with ribosomal protein L23 (gray ribbon). (D) Surface representation of eIF6 interacting with ribosomal protein GTPBP4 (gray ribbon). (E,F) Effect of the F34L mutation: red solid disks represent clashes between the two rotamers of L34 and neighboring residues.*

5.2 Distribution of germline variants related to the development of hematological abnormalities in 16 Patients affected with SDS

In this study, we reviewed all available hematologic data, including medical records, CBCs and bone marrow smears, to describe the hematologic outcome. Different types of variant filtering were used to identify the distribution of germline variants related to the development of neutropenia, bone marrow hypocellularity, thrombocytopenia and haematological malignancies, including MDS and AML using eVai software as described in method section (3.4.2).

5.2.1 Hematological characteristics of patients

Neutropenia was found in all patients at the disease diagnosis. The vast majority of patients (N=13,81%) had marrow hypocellularity. On the other hand, thrombocytopenia was found in 12 cases. To this date (September 2022), only one patient (UPN 45) developed MDS and aplastic anemia. Similarly, one patient (UPN 24) had anemia, and one patient (UPN 42) had been diagnosed with Hodgkin lymphoma. Leukopenia and decreased circulating antibody levels were found in UPN 85 (Figure 5.4).

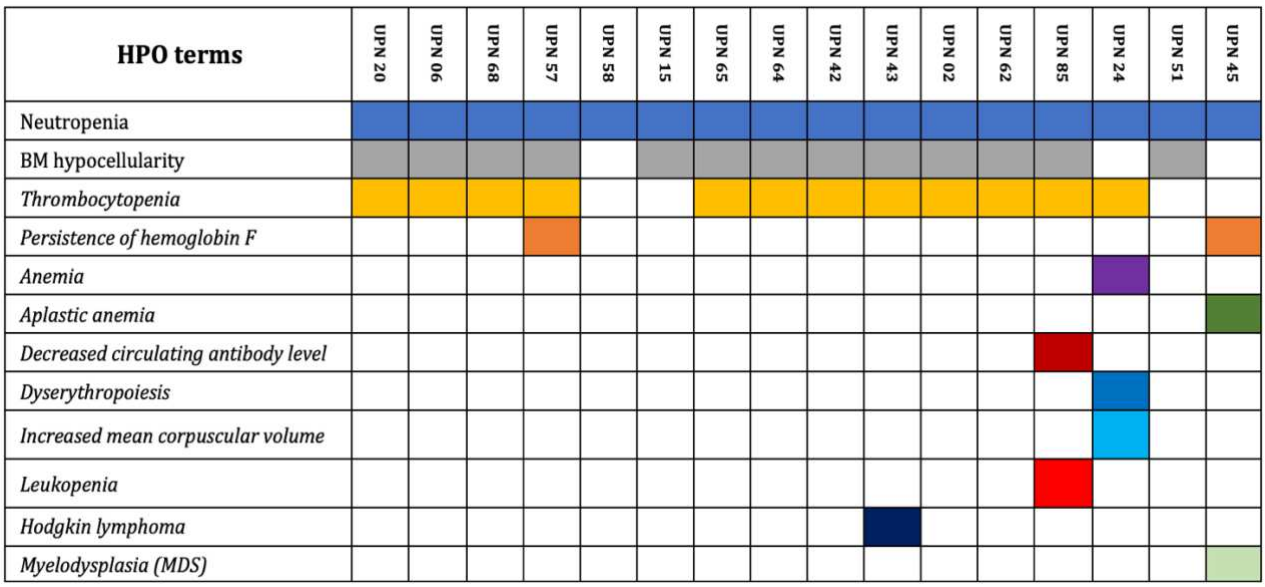


Figure 5.4. *The distribution of hematological complications found in 16 SDS patients*

The neutrophils count mean is 1132 cells / mcL, SD is 777, and the range is 280 to 3110. UPN 45 has the lowest neutrophil count (280 cells/mcL). The mean platelet count is 158,000 (cells/mcL). The bone marrow status of the patients was varied; eight patients (50%) had no or mild bone marrow dysfunction, whereas five (31.25%) and three

(18.75%) patients had moderate and severe bone marrow dysfunction, respectively (Table 5.3).

Table 5.3. *ANCs, platelet counts and bone marrow status for 16 SDS patients.*

UPNs	ANC (Cells/ mcL)	Platelet counts (Cells/ mcL)	Bone marrow status
UPN 58	280	153000	Severe
UPN 20	500	86000	Moderate
UPN 57	530	113000	Moderate
UPN 24	750	71000	Moderate
UPN 2	900	146000	Moderate
UPN 85	900	99000	Mild
UPN 42	972	129000	Severe
UPN 65	1040	132000	Mild
UPN 43	1095	95000	Mild
UPN 45	1300	269000	Severe
UPN 15	1340	207000	Mild
UPN 64	1390	136000	Moderate
UPN 62	1730	113000	Mild
UPN 68	1907	121000	Mild
UPN 51	2409	199000	Mild
UPN 6	3110	81000	Mild

All patients were grouped according to their hematological information (Table 5.4).

Table 5.4. *Groups of patients according to their hematological data.*

1. Neutropenia groups	
Group 1.1: Patients with severe neutropenia (280-750 cells/mL)	UPNs 58, 20, 57 and 24
Group 1.2: Patients with moderate neutropenia (900- 1390 cells/ mL)	UPNs 2, 85, 42, 65,43, 45, 15 and 64
Group 1.3: Patients with Mild neutropenia (1730-3110 cells/mL)	UPNs 62, 68, 51 and 6
2. Bone marrow status groups	
Group 2.1: Patients with severe bone marrow status	UPNs 58, 42 and 45
Group 2.2: Patients with moderate bone marrow status	UPNs 20, 57, 64, 2 and 24
Group 2.3: Patients with mild bone marrow status	UPNs 6, 68, 15, 65, 43, 62, 85 and 51
3. Thrombocytopenia groups	

Group 3.1: Patients with reduced platelets count (71000- 100000 cell/ mL)	UPNs 24, 6, 20, 43 and 85
Group 3.2: Patients with normal platelets count (more than 100000 cells/ mL)	The rest of the patients

5.2.2 Analysis of variants in genes related to the development of MDS and/ or AML

5.2.2.1 Results of filter 2.1 (Variants with an eVai pathogenicity score ≥ 0)

A total of 515 germline genetic variations, including nucleotide substitutions (N = 477) and small insertions or deletions (N = 29) were identified in 87 genes. No larger chromosomal deletions were identified. Approximately, 29% of variants were exonic. Only 25 variants were inherited in a homozygous status. Based on the ACMG classifications, 81% of variants were classified as VUS, 18% were classified as likely benign, and only 1% were classified as pathogenic or likely pathogenic (Figure 5.5).

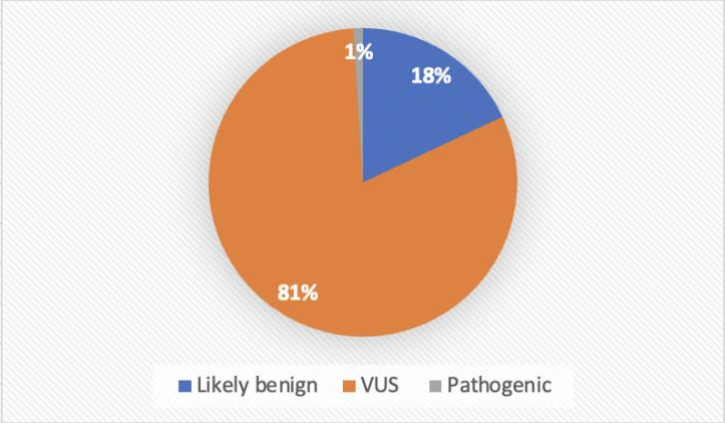


Figure 5.5. *The distribution of variants resulting from filter 2.1 according to the ACMG classifications.*

The mean number of variants per patient is 38.75, SD is 7.2 (22 to 56). UPN 45, UPN 85 and UPN 51 have the highest number of variants, while UPN6 and UPN 68 have the lowest number. Three patients (UPNs 2, 42 and 20) shared the same number of variants (Figure 5.6).

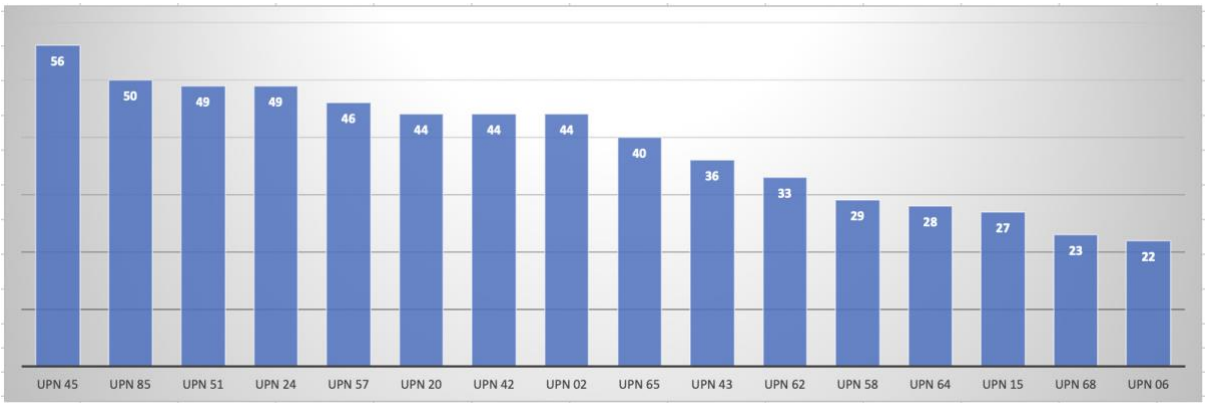


Figure 5.6. *Number of variants per patient resulting from filter 2.1*

We found that *TET2, ATM, BCR, CLCN7, BRCA1, CBL, FANCA, PAX5, TBXAS1, NF1* and *JAK2* genes were the most commonly mutated genes among our SDS cohort. *TET2* was found to be mutated in 11 patients (68.75%), followed by *ATM* (N=10, 62.5%), *BCR* (N=10, 62.5%), *CLCN7*, (N=10, 62.5%), *BRCA1* (N=9, 56.25%), *CBL* (N=9, 56.25%), *FANCA* (N=9, 56.25%), *PAX5* (N=9, 56.25%), *TBXAS1*(N=9, 56.25%), *JAK2* (N=9, 56.25%) and *NF1* (N=8, 50%) (Figure 5.7).

Figure 5.7. *The most commonly mutated genes per patient resulting from filter 2.1*

5.2.2.2 Results of filter 2.2 (Coding variants with MAF ≤ 0.05)

We identified 268 gene mutations in these 16 patients (median, 27 variants per patient; range, 16 to 66). We found 25 delins, 216 missense, 1 start loss, 3 stop gained, and 23 splicing variants (Figure 5.8). About 65% of variants were classified as VUS, and 2% were classified as pathogenic or likely pathogenic.

The variants in the *BRCA2* (c.7825G>A), and *SEC23B* (c.40C>T) genes were classified as pathogenic and observed in UPN45, and UPN24, respectively. Two variants were classified as likely pathogenic in the *KMT2A* (c.5336T>G, and c.10663G>A), found in UPN 45 and UPN 43. In UPN43 also, we identifed another likely pathogenic variant (c.1781T>G) in the *SEC23B.*

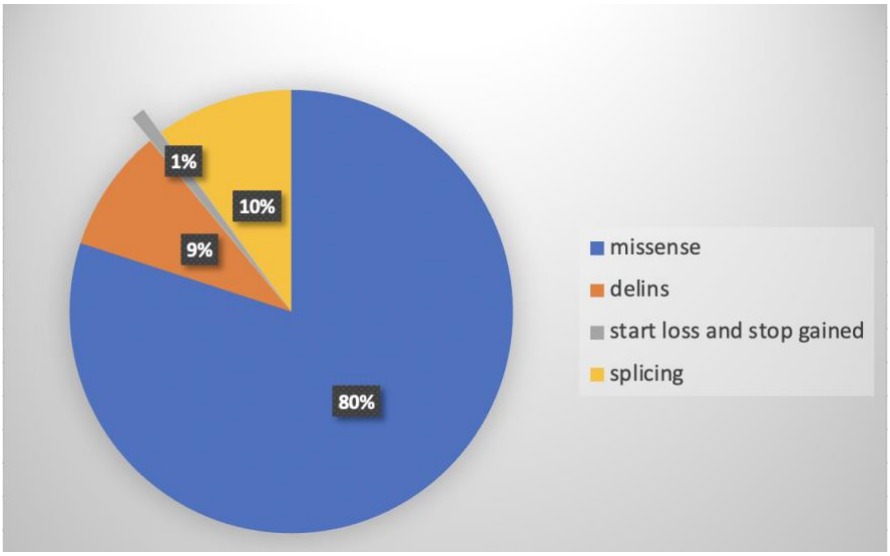


Figure 5.8. *The percentages of genetic variations effects found in filter 2..2*

In line with previous results of filter 2.1, we found that UPN 45 carries significantly the highest number of variants (N=66). In contrast, the lowest number of variants (N=16) belongs to UPN 62. UPNs 65, 6, 43 and UPN 85 shared the same number of variants (N=43) and occupied the second rank (Figure 5.9).

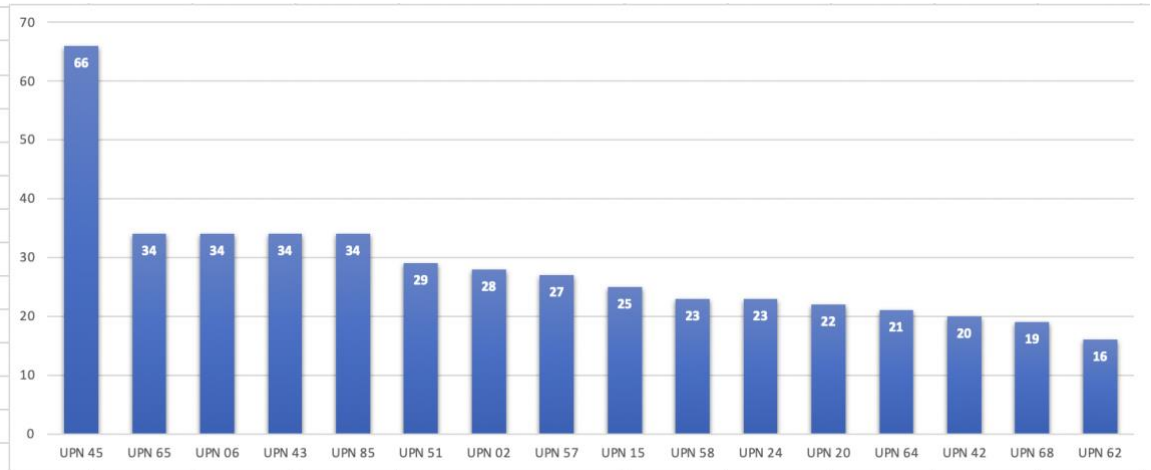


Figure 5.9. *The number of variants per patient identified by filter 2.2*

The most frequently mutated genes were *TBXAS1* (in 7 of 16 patients; 50%), *CUX1, FANCA, RTEL1, SAMD9L, SEC23B,* and *TET2* were identified in 6 patients (37.5%). Additionally, in 5 patients (31, 25%), we found variants in *ATM, BRCA1, CTCFL, GP6, KMT2D, SLX4,* and *XRCC4.* Variants in *ERCC6L2 and KLF1* were detected in 25% of patients. However, The *TET2* gene has the highest number of variants (N=7), followed by *TBXAS1* (N=5), *SEC23B* (N=5) and *BRCA1* (N=5) (Table 5.5).

Table 5.5. *Number of patients, and variants found in the most frequently genes resulting from filter 2.2*

Gene	No. Patients	No. Variants
TBXAS1	8	5
CUX1	6	3
FANCA	6	2
RTEL1	6	3
SAMD9L	6	2
SEC23B	6	5
TET2	6	7
ATM	5	5
BRCA1	5	5
CTCFL	5	2
GP6	5	2
KMT2D	5	4
SLX4	5	3
XRCC4	5	3
ERCC6L2	4	4
KLF1	4	2

The eVai pathogenicity score mean for variants in the most frequently mutated genes is 1.36. Variants in *BRCA1* and *SEC23B* have the highest pathogenicity score mean (3). However, variants of *SLX4, CUX1, ERCC6L2, TBXAS1, CTCFL*, and *SAMD9L* all shared the same mean pathogenicity score (1) (Figure 5.10).

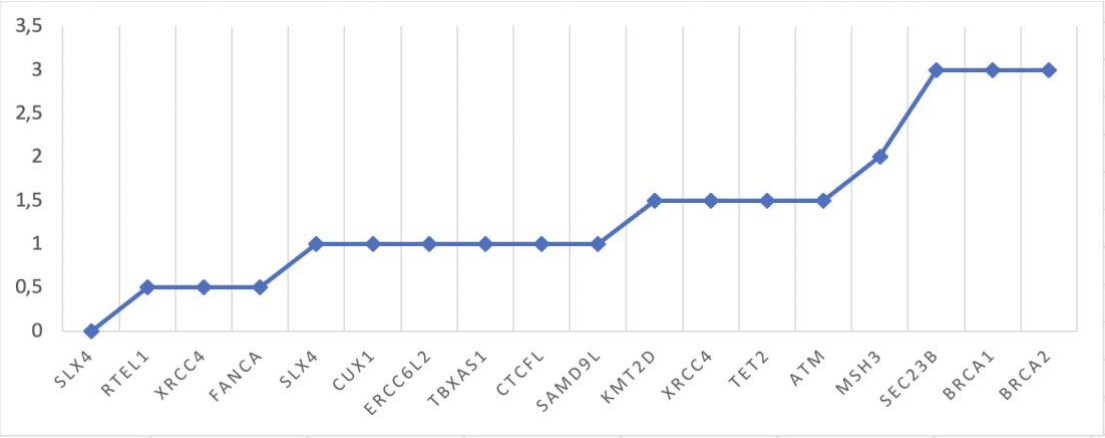


Figure 5.10. *The eVai pathogenicity mean for variants found in the most frequently mutated genes resulting from filter 2.2*

All variants, in the most commonly mutated genes, were listed in table 5.6 according to their effect, zygosity, MAF and different in silico prediction tools including eVai, SIFT, mutation taster (MT), mutation assessor (MA), M-CAP, REVEL, and ACMG.

Table 5.6. *Variants found in the most commonly mutated genes resulting from filter 2.2*

Gene	HGVS_Coding	HGVS_Protein	Effect	Zyg	MAF	*In silico* prediction tools						
						eVai	SIFT	MT	MA	M-CAP	REVEL	ACMG
BRCA1	c.314A>G	p.Tyr105Cys	missense	het	0.007	4.0	B	D	B	D	D	VUS
	c.4956G>A	p.Met1652Ile	missense	het	0.018	0.5	B	B	B	B	D	VUS
	c.1486C>T	p.Arg496Cys	missense	het	0.002	0.5	B	B	B	D	N/A	LB
	c.3119G>A	p.Ser1040Asn	missense	het	0.012	2.0	D	D	B	B	D	VUS
	c.1067A>G	p.Gln356Arg	missense	het	0.047	2.0	D	D	B	B	B	LB
BRCA2	c.7825G>A	p.Gly2609Ser	missense	het	0.001	6.5	D	D	D	D	D	P
	c.4258G>T	p.Asp1420Tyr	missense	het	0.041	1.5	B	B	B	B	B	LB
	c.5744C>T	p.Thr1915Met	missense	het	0.050	0.5	B	B	B	D	B	LB
TBXAS1	c.1345G>A	p.Glu449Lys	missense	het	0.010	0.5	B	B	B	B	N/A	LB
	c.541C>T	p.Pro181Ser	missense	het	0.020	0.5	N/A	B	B	B	N/A	LB
	c.1066C>G	p.Leu356Val	missense	het	0.030	0	B	B	B	B	B	LB
	c.1349C>A	p.Thr450Asn	missense	het	0.014	0	B	B	B	B	B	LB
	c.-317-3delT	N/A	Splicing	het	0.008	1	B	B	D	B	B	LB
CUX1	c.1627A>G	p.Ile543Val	missense	het	0.027	0	B	B	B	B	B	LB
	c.1630A>C	p.Lys544Gln	missense	het	0.005	0	D	D	B	B	N/A	LB
	c.1675G>A	p.Gly559Ser	missense	het	0.001	0	B	D	B	N/A	N/A	VUS
FANCA	c.2574C>G	p.Ser858Arg	missense	het	0.006	0	B	B	B	N/A	B	LB
	c.2151G>T	p.Met717Ile	missense	het	0.002	0	B	B	B	B	B	LB
RTEL1	c.2051G>A	p.Arg684Gln	missense	het	0.020	0.5	B	N/A	B	B	B	LB
	c.2785G>A	p.Ala929Thr	missense	het	0.037	0	B	B	B	N/A	B	LB
	c.3590G>C	p.Gly1197Ala	missense	het	0.003	0	B	B	B	B	N/A	LB
SAMD9L	c.866T>C	p.Phe289Ser	missense	het	0.020	0	B	B	D	B	B	VUS
	c.1565C>T	p.Ala522Val	missense	het	0.013	0	B	D	B	N/A	B	VUS
SEC23B	c.770C>T	p.Thr257Ile	missense	het	0.020	0	B	B	B	B	B	LB
	c.1276G>A	p.Val426Ile	missense	het	0.040	0.5	B	D	B	B	N/A	VUS
	c.1781T>G	p.Val594Gly	missense	het	0.001	2.5	D	D	D	D	D	LP
	c.40C>T	p.Arg14Trp	missense	het	0.001	3	D	D	D	D	D	P
	c.490G>T	p.Val164Leu	missense	het	0.030	0	D	B	B	B	D	LB
TET2	c.1064G>A	p.Gly355Asp	missense	het	0.020	0.5	B	B	B	B	B	LB
	c.5152G>T	p.Val1718Leu	missense	het	0.004	0	B	B	B	B	B	LB
	c.1907T>C	p.Val636Ala	missense	het	0.001	1	B	B	B	D	B	VUS
	c.2599T>C	p.Tyr867His	missense	het	0.006	1	D	D	B	B	B	LB
	c.5167C>T	p.Pro1723Ser	missense	het	0.001	0	B	D	B	B	B	LB
	c.2996A>G	p.Glu999Gly	missense	het	0.000	2.5	B	D	B	B	D	VUS
	c.100C>T	p.Leu34Phe	missense	het	0.001	0.5	D	D	B	B	B	VUS
ATM	c.1744T>C	p.Phe582Leu	missense	het	0.003	0	B	B	B	B	N/A	LB
	c.3161C>G	p.Pro1054Arg	missense	het	0.001	0.5	D	D	B	B	B	LB
	c.1066-6T>G	N/A	Splicing	het	0.003	1	N/A	N/A	N/A	N/A	N/A	VUS
	c.8786+8A>C	N/A	Splicing	het	0.020	0	N/A	N/A	N/A	N/A	N/A	LB
	c.2572T>C	p.Phe858Leu	missense	het	0.008	0	B	B	B	B	B	LB
CTCFL	c.1339A>G	p.Met447Val	missense	het	0.005	1	B	B	N/A	B	B	LB
	c.563A>T	p.Lys188Met	missense	het	0.043	1	B	D	B	B	B	LB
GP6	c.614C>A	p.Thr205Asn	missense	het	0.001	1	B	B	B	N/A	B	VUS
	c.1495G>A	p.Gly499Ser	missense	het	0.020	0	D	B	B	B	D	LB
KMT2D	c.305G>A	p.Ser102Asn	missense	het	0.002	0	B	B	B	B	D	VUS
	c.10045A>G	p.Met3349Val	missense	het	0.010	0	B	B	B	B	B	LB
	c.7670C>T	p.Pro2557Leu	missense	het	0.008	0	B	D	B	B	B	LB
	c.3296G>A	p.Gly1099Glu	missense	het	0.001	0	D	B	B	B	B	VUS
XRCC4	c.401T>C	p.Ile134Thr	missense	het	0.023	0.5	B	B	B	N/A	B	VUS
	c.739G>T	p.Ala247Ser	missense	het	0.050	0	B	B	B	B	B	LB
	c.166G>A	p.Ala56Thr	missense	het	0.007	1	D	D	B	D	B	VUS
ERCC6L2	c.8C>A	p.Pro3Gln	missense	het	0.001	0	B	B	B	B	D	VUS
	c.2486C>T	p.Thr829Ile	missense	het	0.021	0	B	N/A	B	N/A	B	LB
	c.2236C>G	p.Leu746Val	missense	het	0.050	0	B	B	B	B	B	LB
	c.4403T>C	p.Met1468Thr	missense	het	0.040	0	B	B	B	B	N/A	LB
KLF1	c.115A>C	p.Met39Leu	missense	het	0.012	0	B	D	B	B	D	VUS
	c.544T>C	p.Phe182Leu	missense	het	0.010	1	B	D	B	D	D	VUS

Zyg: zygosity, **MAF**: minor allele frequency, **SIFT**: Sorting Intolerant from Tolerant, **MT**: Mutationt Taster, **MA**: Mutation Assessor, **ACMG**: American College of Medical Genetics guidelines, **B**: benign, **LB**: Likely Benign, **P**: Pathogenic, **LP**: Likey pathogenic, **D**: Damaging

We checked the most frequently mutated genes according to their genetic pathway using the PathCards database. We found that 34% of the genes (*MSH3, RTEL1, SLX4, XRCC4, FANCA and ERCC6L2*) belong to the DNA repair pathway. About 16% of genes (*SLX4*, *XRCC4*, and *CUX1*) play roles in the signaling pathway. Similarly, 16% of genes were involved in transcriptional regulation (*TET2, CTCFL,* and *KMT2D*) and BRCA1 pathways like the *ATM*, *BRCA1*, and *BRCA2* genes. The metabolism pathway included two genes (TBXAS1 and SEC23B). One gene (*SAMD9L*) was classified as a tumor suppressor. Figure 5.11 shows the distribution of most mutated genes, found in filter 2.2, according to their genetic pathways.

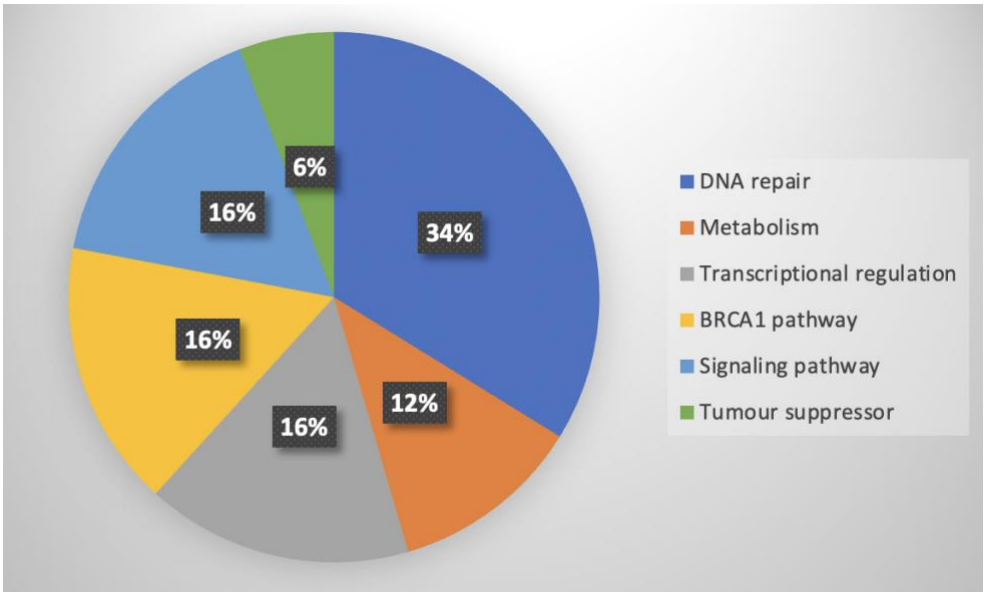


Figure 5.11. *The percentages of genetic pathways found in the most frequently mutated genes resulting from filter 2.2*

We found that all patients, except UPN 2, are carrying variants in genes involved in the DNA repair pathway. Interestingly, among all of the patients, only UPN45 was found to be carrying variants in all selected pathways. UPN 57 has variants in genes only related to DNA repair and transcriptional regulatory pathways (Figure 5.12).

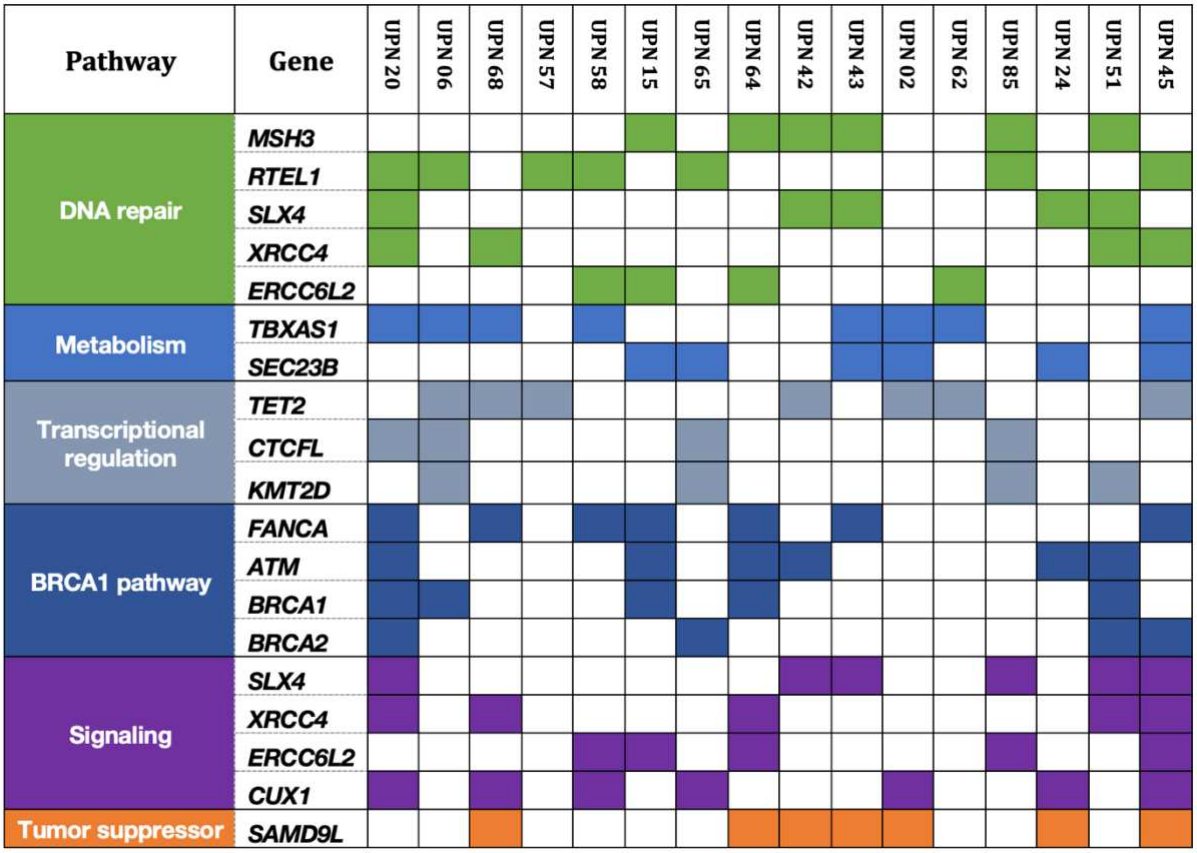


Figure 5.12. *The distribution of patients and genes according to the genetic pathways*

5.2.3 Analysis of variants in genes related to the development of neutropenia

5.2.3.1 Results of filter 3 (Variants with an eVai pathogenicity score ≥ 0)

A total of 195 germline genetic variations in 55 genes were detected. Only five variants were inherited in a homozygous status. The majority of variants were intronic (N=124, 63.5%). Based on the ACMG classifications, 89% of variants were classified as VUS, 9% were classified as likely benign, and only 2% were classified as pathogenic or likely pathogenic (Figure 5.13).

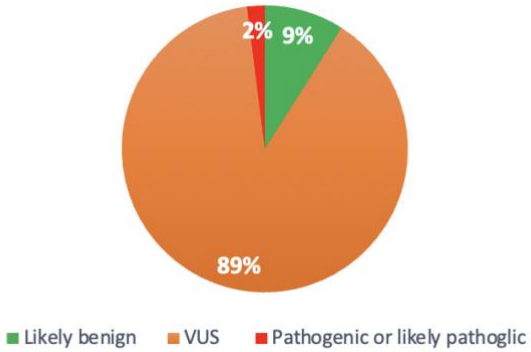


Figure 5.13. *The distribution of variants resulting from filter 3 according to the ACMG classifications.*

The mean number of variants per patient is 48, SD is 8.3,(range, 33- 62). UPNs 24, 57 and 20 have the highest number of variants (N=62, 57 and 56, respectively), while UPN 45 and 15 have the lowest number (N=39 and 33, respectively). Three patients (UPNs 85, 42 and 58) shared the same number of variants (N=55) (Figure 5.14).

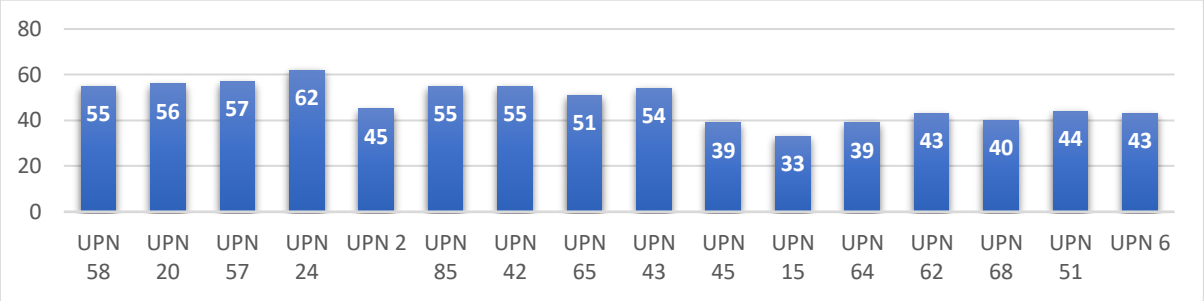


Figure 5.14. *The number of variants per patient identified by filter 3.*

We found that *VPS13B* and *CSF3R* were the genes most commonly mutated, and their variants were observed to be more prevalent in group 1.1 (patients with severe neutropenia) and group 1.2 (patients with moderate neutropenia) (Figure 5.15).

Group 1.1				Group 1.2					Group 1.3						
280-750 cell/mL				900- 1095 Cell/ mL					1300-3110 Cells/mL						
UPN 58	UPN 20	UPN 57	UPN 24	UPN 2	UPN 85	UPN 42	UPN 65	UPN 43	UPN 45	UPN 15	UPN 64	UPN 62	UPN 68	UPN 51	UPN 6
CSF3R	CSF3R	CSF3R			CSF3R	CSF3R	CSF3R	CSF3R			CSF3R		CSF3R	CSF3R	
VPS13B	VPS13B	VPS13B	VPS13B	VPS13B	VPS13B	VPS13B	VPS13B	VPS13B			VPS13B	VPS13B			

Figure 5.15. *The distribution of VPS13B and CSF3R variants according to neutropenia groups.*

VPS13B was mutated in 11 of the 16 patients (68.75%), followed by *CSF3R* (N=10, 62.5%) and *SAMD9L* (N=9, 56.25%). Variants in four genes (*FUT8, MMAB, SLC35A1* and *FANCA*) were identified in 6 patients. Variants observed in a smaller number of cases are also listed in figure 5.16.

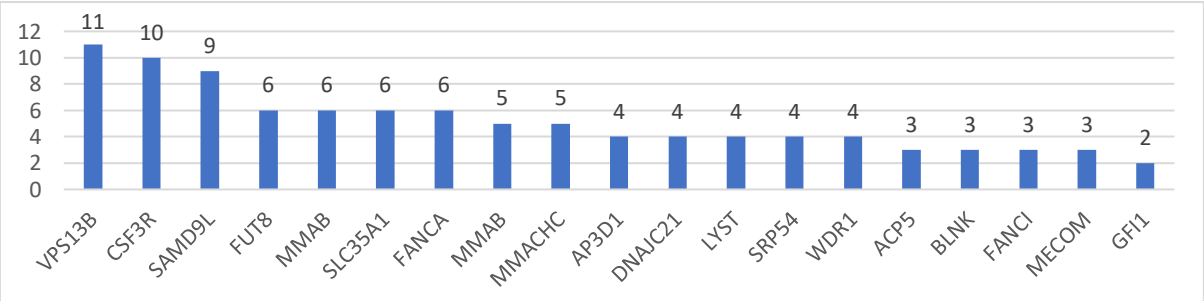


Figure 5.16. *The most commonly mutated genes per patient resulting from filter 3.*

Most *VPS13B* variations are intronic (11 out of 14). According to the ACMG, 10 variants were classified as VUS, and 4 were classified as likely benign. All variants (N=6) detected in the *CSF3R* gene were missense. Only one variant was classified as likely pathogenic and 5 were classified as VUS. All *CSF3R* variants have MAF less than 0.05. Additionally, we identified 2 intronic variants in the *SAMD9L* gene distributed in 9 probands.

To determine the relationship between the absolute neutrophil count and the number of variants in each patient, we used Pearson correlation using the SPSS software. The absolute neutrophil count (ANC) and the number of variants were found to be significantly negatively correlated (R=-0.601, $p < 0.05$) (figure 5.17).

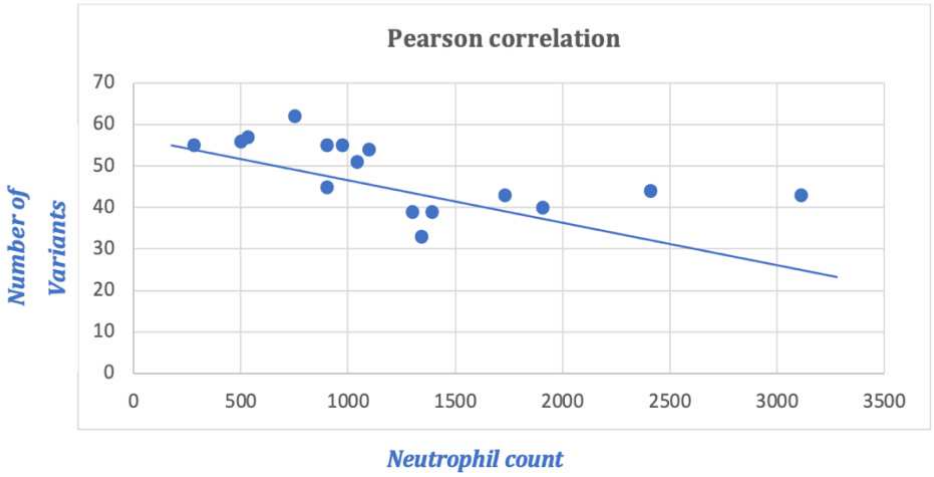


Figure 5.17. *Pearson correlation scotter plot showing the negative correlation between the number of variants per patients and the ANC*

In figure 5.18, we report the MAF for quoted genes (reported as the mean of all variants observed for each gene); *CSF3R* variants have the lowest MAF mean (range, 0,0001-0.004), whereas *AP3D1* has the highest one (0,451).

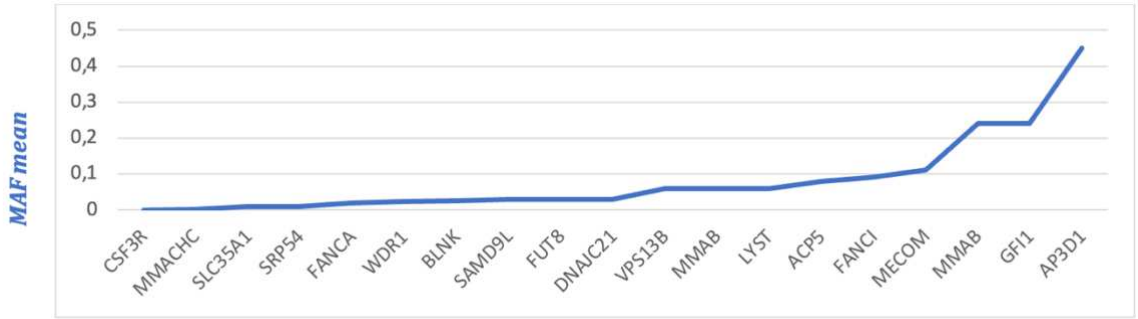


Figure 5.18. *Shows in a scatter plot, the MAF mean of the genes listed above.*

In figure 5.19, we include the mean eVai pathogenicity score for each gene. Variants in *CSF3R, MMACHC* and *FANCA* have the highest pathogenicity score mean (2.5).

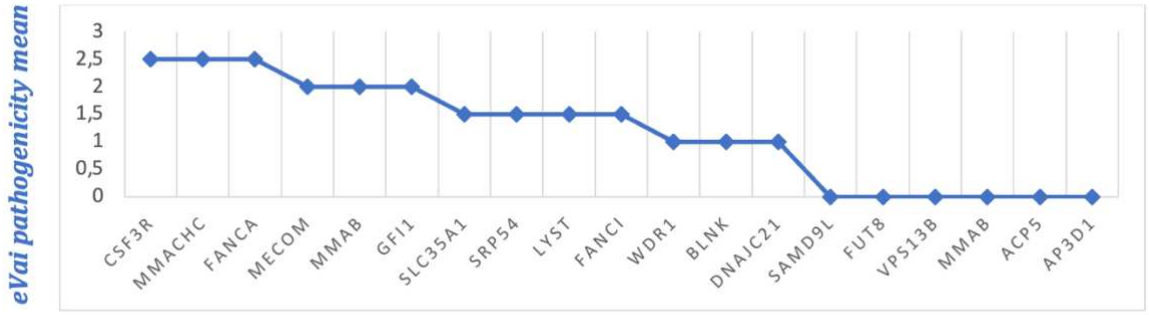


Figure 5.19. *Shows in a scatter plot, the eVai pathogenicity score mean of the genes listed above.*

The most commonly mutated genes will be discussed here:

- ***VPS13B***

Pathogenic variants in the *VPS13B* gene cause Cohen syndrome (OMIM #216550), an autosomal recessive disorder. A total of 14 genetic variations in this gene were inherited in heterozygous status. Only three missense variants (c.7678G>A, c.1768G>A and c.2518G>A) have been identified, and all the remaining variants were intronic. The variants c.7678G>A and c.1768G>A were classified as likely benign, whereas the 2518G>A variant was classified as VUS, but with an eVai pathogenicity score of 2.5. One patient (UPN 24) carried two missense variants (c.2518G>A and c.7678G>A). The patient did not present any significant clinical relevance to Cohen syndrome; it should be noted, that at the time of writing parental status is not known.

- ***CSF3R***

CSF3R mutations have been associated with severe congenital neutropenia 7 (OMIM, 617014, AR) and hereditary neutrophilia (OMIM,162830, AD). In our patients, six heterozygous missense substitutions were identified. The variant (c.1853C>T) was classified as pathogenic with an eVai pathogenicity score of 4.5, while the remaining five (c.2483A>G, c.2167C>T, c.1608G>C, c.1807G>T, and c.2391G>T) were classified as VUS. All variants' minor allele frequencies were found to be less than 0.01%. Notably, *CSF3R* variants had the lowest mean MAF and highest mean pathogenicity scores of all genes related to neutropenia (Figures 6.15 and 6.16). Three out of four patients with severe neutropenia (UPNs 58, 57 and 20) carried biallelic variants in the *CSF3R* gene. Information regarding the *CSF3R* variants is listed in Table 5.7.

Table 5.7. *Information about the CSF3R found in our group of patients*

UPNs	HGVS_Coding	HGVS_Protein	Zyg	MAF	*In silico prediction tools*						
					eVai	SIFT	MT	MA	M-CAP	PROVEAN	ACMG
UPNs 58 and 65	c.1853C>T	p.Thr618Ile	het	0.0001	4.5	D	D	D	D	D	P
UPNs 42 and 43	c.2391G>T	p.Leu797Phe	het	0.0011	1	B	D	B	D	B	VUS
UPNs 85 and 20	c.2483A>G	p.His828Arg	het	0.004	2	D	B	D	B	-	VUS
UPNs 57 and 68	c.2173C>G	p.Gln725Glu	het	0.0012	2	B	D	B	D	D	VUS
UPNs 57 and 20	c.1757T>C	p.Val586Ala	het	0.0001	2.5	B	D	D	D	D	VUS
UPNs 58, 51, 64	c.1484G>C	p.Arg495Thr	het	0.0125	1.5	B	B	B	-	D	VUS

- **Single patient observations, cases with severe neutropenia (group 1.1).**

- UPN 58

UPN 58 has the lowest ANC (250 cells/L) and has a relatively high number of variants (N=55). In the patient, in addition to the two variants (c.1853C>T and c c.1484G>C) in the CSF3R gene, we identified one variant (c.107G>C) in the *GFI1* gene. This variant was classified as VUS and have a MAF of less than 0.05% (Table 5.8). Similar to the *CSF3R* gene, *GFI1* variants have been linked to severe congenital neutropenia 2, but in an autosomal dominant inheritance (OMIM: 613107).

Table 5.8. *Information about the variant found in the GFI1 in UPN58*

HGVS_Coding	HGVS_Protein	Zyg	MAF	*In silico prediction tools*						
				eVai	SIFT	MT	MA	M-CAP	PROVEAN	ACMG
c.107G>C	p.Ser36Thr	Het	0.00013	3	D	B	D	D	B	VUS

- UPN 24

The patient has the highest number of variants (N=62) and shows a reduced ANC (750 cells/L). Two missense variants (c.2518G>A and c.7678G>A) in the *VPS13B* gene have been identified. We also identified a rare variant in the *MDM4* gene (c.1162C>G). The variant is classified as VUS with a MAF of 0.0064. According to OMIM, the *MDM4* gene is associated with bone marrow failure syndrome 6, autosomal dominant (OMIM:602704). Additionally, A VUS missense variation (c.2185T>A) in the FANCI gene has also been found, with an eVAI pathogenicity score of 3 (Table 5.9).

Table 5.9. *Information about the variant found in UPN 24*

Gene	HGVS_Coding	HGVS_Protein	Zyg	MAF	*In silico prediction tools*						
					eVai	SIFT	MT	MA	M-CAP	PROVEAN	ACMG
MDM4	c.1162C>G	p.Pro388Ala	het	0.0064	2	D	B	D	D	-	VUS
FANCI	c.2185T>A	p.Phe729Ile	het	0.0003	3	D	D	-	D	D	VUS

- **UPN 20**

The patient has a high number of variants (N=57) and a low ANC (500 cells/L). Two rare missense variants (c.2391G>T and c.2483A>G) in the *CSF3R* gene in addition to a rare variant (c.196G>A) in the 40S ribosomal protein S19 (RPS19) have been identified. The variant c.196G>A is a rare one (MAF=0.0059), classified as VUS with an eVai pathogenicity score of 2 (Table 5.10). Mutations in *RPS19* have been associated with Diamond-Blackfan anemia-1, an autosomal dominant condition (OMIM:105650).

Table 5.10. *Information about the variant found in UPN 20*

Gene	HGVS_Coding	HGVS_Protein	Zyg	MAF	*In silico prediction tools*						
					eVai	SIFT	MT	MA	M-CAP	PROVEAN	ACMG
RPS19	c.196G>A	p.Ala66Thr	het	0.0059	2	D	B	D	D	D	VUS

- **UPN 57**

Like other patients in group 1, UPN 57 has a high number of variants (N=57) and low ANC (530 cells/L). In addition to biallelic variants in the *CSF3R* gene (Table 5.7), the patient carries a missense variant (c.133A>G) in the 40S ribosomal protein S7 (RPS7). The *RPS7* variant is classified as VUS with an eVai pathogenicity score of 2.5. Notably, the heterozygous mutations in the *RPS7* cause the condition of Diamond-Blackfan anemia-8 (OMIM:612563).

Table 5.11. *Information about the variant found in UPN 57*

Gene	HGVS_Coding	HGVS_Protein	Zyg	MAF	*In slico prediction tools*						
					eVai	SIFT	MT	MA	M-CAP	PROVEAN	ACMG
RPS7	c.133A>G	p.Ala66Thr	het	N/A	2.5	D	D	D	B	-	VUS

5.2.4 Analysis of variants in genes related to the development of bone marrow hypocellularity

5.2.4.1 Results of filter 4 (Variants with an eVai pathogenicity score ≥ 0)

After applying filter 4, we obtained 50 variants in 16 genes. Half of the variants (N=24) were exonic, and only one variant in the *PRF1* gene was inherited in homozygous status. The vast majority of variants were classified as VUS (N=38, 76%), one variant in the *LBR* gene was classified as pathogenic, and 11 variants (13%) were classified as likely benign. No benign variants were identified.

The mean number of variants per patient is 43,8, SD is 8,2 (33-57). UPN65 and UPN20 carry the highest number of variants, conversely, UPN42 and UPN51 have the lowest number. Three patients (UPNs 57,2 and 15) are harboring the same number of variants (Figure 5.20).

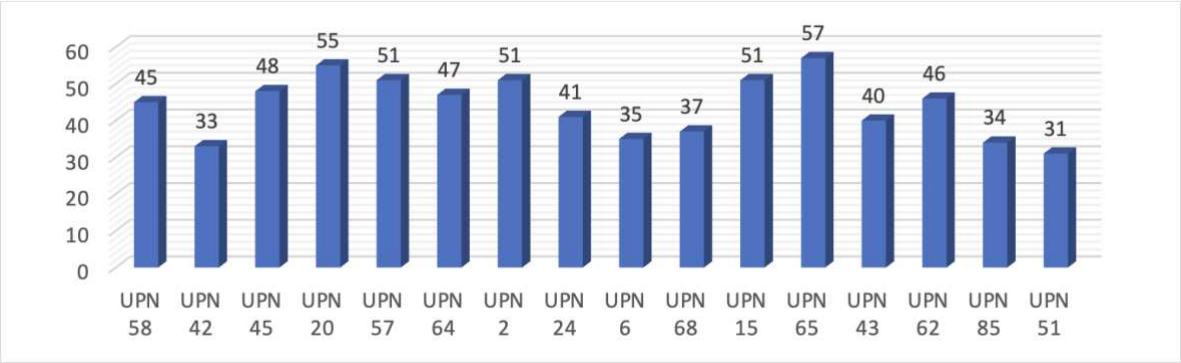


Figure 5.20. *The number of variants per patient identified by filter 4.*

Variants in the *PRF1* and *CLCN7* were more prevalent and found in 9 patients. *TERT, NHP2,* and *VPS33A* variants were identified in 4 patients, while, variants in *DNAJC21, FANCI, LBR, TBXAS1* and *WDR19* were detected in 3 patients (Figure 5.21).

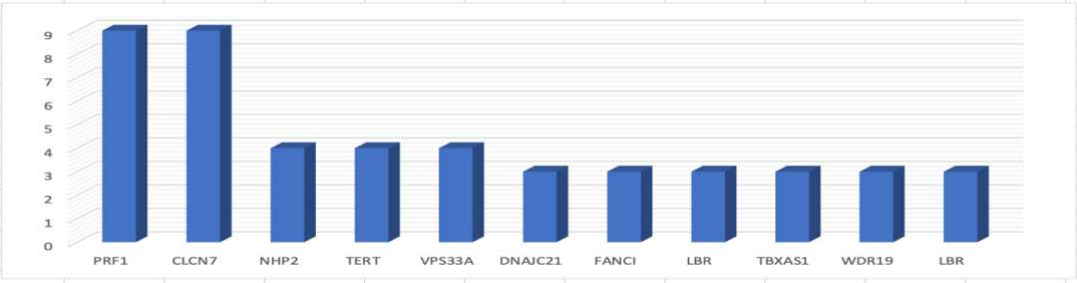


Figure 5.21. *The most commonly mutated genes per patient resulting from filter 4.*

Here, the most frequently mutated genes will be discussed:

- ***PRF1***

In our cohort, we identified 3 missense variants (c.272C>T, c.755A>G, and c.632C>T) found in 9 patients. Only variant c.272C>T was inherited in homozygous status in UPN 57 and UPN 20. All variants, except c.632C>T (likely benign), were classified as VUS and had a MAF of less than 0.05 (Table 5.12). It is interesting to note that two out of three patients with severe bone marrow hypocellularity (group 2.1) have biallelic mutations in the PRF1 either in homozygous (UPN58) or compound heterozygous form (UPN45).

Table 5.12. *Data about variants found in the PRF1 gene*

UPNs	HGVS_Coding	HGVS_Protein	Zyg	MAF	*In silico prediction tools*						
					eVai	SIFT	MT	MA	M-CAP	PROVEAN	ACMG
UPNs 65, 64, 24 and 45	c.272C>T	p.Ala91Val	het	0.020	1.5	B	D	B	D	D	VUS
UPNs 57, 20 and 58	c.272C>T	p.Ala91Val	hom	0.020	1.5	B	D	B	D	D	VUS
UPNs 51 and 45	c.755A>G	p.Asn252Ser	het	0.050	1	D	B	D	B	B	VUS
UPN42	c.632C>T	p.Ala211Val	het	0.021	0.5	D	B	D	B	B	LB

- **CLCN7**

All the variants identified in the chloride channel 7 (*CLCN7)* were intronic, classified as VUS, and inherited in heterozygous status in 9 patients. Autosomal-dominant osteopetrosis type II (ADOII) is most often caused by mutation of the *CLCN7* gene leading to impaired bone resorption (OMIM:602727).

5.2.5 Analysis of variants in genes related to the development of thrombocytopenia

5.2.5.1 Results of filter 5 (Variants with an eVai pathogenicity score ≥ 0)

As a result of filter 5, we identified 245 variants in 66 genes, most of them (N=202) were classified as VUS, and 40 variants were classified as likely benign. Four variants in the *CD36, VWF*, *ADAMTS13* and *SRC* genes are classified as likely pathogenic. Of the 245 variants, only 63 were missense and 8 variants were inherited in homozygous status.

The mean number of variants per patient is 29, SD is 7.5 (16-44). UPN15 has the highest number of variants (N=44) followed by UPN58 (N=44). In contrast, UPN45 carries the lowest number (N= 15).

UPNs 24, 68 and 65 are carrying the same number of variants (Figure 5.22). No statistically significant correlation between the number of variants and platelet counts has been found using the Pearson correlation test (r= -0.142, P=0.599).

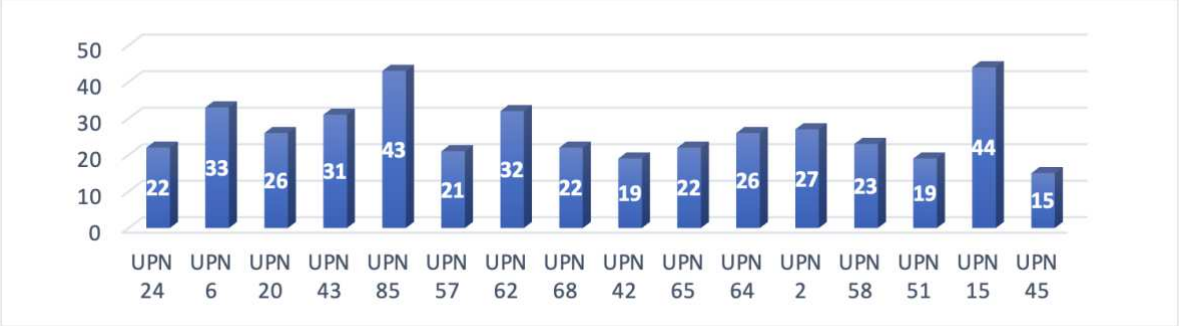


Figure 5.22. *The number of variants per patient identified by filter 5.*

We observed that the genes *TLDC2, MMAB, TNFRSF11A*, and *VWF* were the most frequently mutated. Nine cases have *TLDC2* variations, whereas six cases harbor the *MMAB, TNFRSF11A*, and *VWF* variants. In addition, 5 patients have variants in *ITGB3* and *SLC35A1*. Variants observed in a lower number of cases are also listed in figure 5.23.

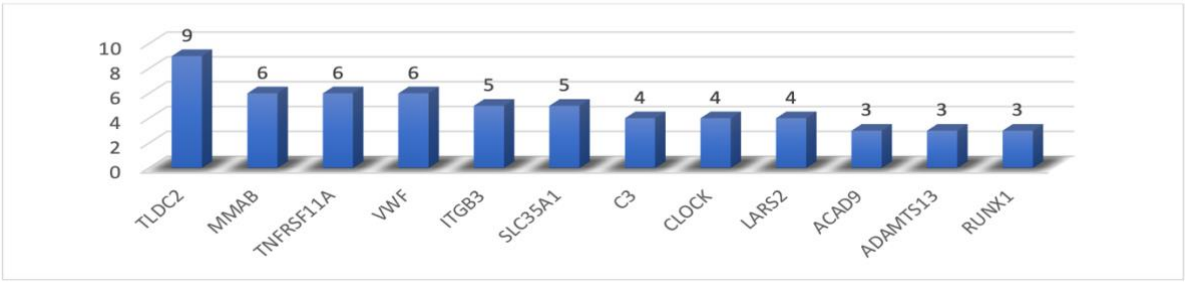


Figure 5.23. *The most frequently mutated genes per patient resulting from filter 5.*

- ***TLDC2, MMAB* and *TNFRSF11A***

All variants found in the *TLDC2 (N=5), MMAB (N=6),* and *TNFRSF11A (N=4)* are intronic with a MAF of more than 0.05. However, the quoted variants have never been published or reported in any case before.

- ***VWF***

Regarding the gene, we identified 6 variants, 3 of which are missenses (c.7636A>T, c.2561G>A and c.3332G>A). The variant c.2561G>A was found in UPN 6 and classified as likely pathogenic with an eVai pathogenicity score of 5.5, whereas the variants c.7636A>T, in UPN85, and c.3332G>A, in UPN62, were classified as likely benign and VUS, respectively.

Importantly, all the missense *VWF* variants were found only in patients with severe thrombocytopenia (group 3.1). Information about variants in the *VWF* genes were listed in table 5.13.

Table 5.13. *Information about the variants in the VWF gene.*

UPNs	HGVS_Coding	HGVS_Protein	Zyg	MAF	*In silico prediction tools*						
					eVai	SIFT	MT	MA	M-CAP	PROVEAN	ACMG
UPN 85	c.7636A>T	p.Asn2546Tyr	het	0.0001	2.5	D	D	D	B	D	VUS
UPN6	c.2561G>A	p.Arg854Gln	het	0.0020	5.5	D	D	D	D	D	LP
UPN20	c.3332G>A	p.Cys1111Tyr	het	N/A	1.5	D	B	D	B	D	VUS

- ***ITGB3, SLC35A1, C3, CLOCK, LARS2, RUNX1and ACAD9***

With the exception of one missense variant (c.976G>A) in the *ACAD9* gene, all the variants in these genes were intronic. The variant c.976G>A was found in UPN85 and classified as VUS with an eVai pathogenicity score of 2 and MAF of 0.017.

- ***ADAMTS13***

Three missense variations (c.577C>T, c.3097G>A and c.949C>T) have been identified in the gene. The variant c.577C>T, with an eVai pathogenicity score of 6, was found in UPN24 and classified as likely pathogenic. The variant is a rare one and has been reported in some cases affected with hereditary thrombotic thrombocytopenic purpura (TTP).

In addition, the variant c.3097G>A found in UPN65, has been also reported in some patients with the same condition. This variant was classified as VUS with a MAF of 0.03. The last one (c.949C>T), found in UPN65, was classified as likely benign and it was never been reported in any case before.

- **Single patient observations, patients with low platelets counts (group 3.1)**

- **UPN 24**

Of all patients, UPN 24 has the lowest platelet count (71000 mcL) and carries a relatively low number of variants (N=22). In addition to the likely pathogenic variant c.577C>T in the ADAMTS13, the patient is harboring another likely pathogenic variant (c.1579G>A) in the *SRC* gene.

The eVai pathogenicity score of the variant is 5.5 and it has not been found in any frequency databases. This variant has been reported in 3 patients affected with thrombocytopenia type 6 in autosomal dominant inheritance.

- **UPN 85**

Compared to other patients, UPN85 had a low platelet count (99000 mcL) and a high number of variants (N=42). In UPN85, we found compound heterozygous rare variants (c.1208T>C, c.1233C>A) in the *SLC46A1* gene. For both variants, the eVai pathogenicity scores are 2.5, MAFs are 0.0003 and 0.0001, respectively, and the ACMG classification is VUS. Homozygous or compound heterozygous mutations in the *SLC46A1* gene are associated with hereditary folate malabsorption (HFM), (OMIM: 611672). Features of thrombocytopenia, neutropenia and leukopenia are frequently observed in patients affected with HFM.

- **UPNs 6, 20 and 43.**

The single variant analysis did not disclose any variation, which could be significantly related to thrombocytopenia.

5.2.6 Summary of filters

Summary of filters used as described in method section (3.4.2).

Table 5.14. *Summary for all filters used.*

Filter No.	**Total No. of variants**	**Exonic variants (N)**	**VUS Variants (N)**	**Likely and pathogenic variants (N)**	**Most frequently mutated genes.**
Filter 2.1 Variants related to MDS and AML eVai pathogenicity score ≥ 0.	515	206	412	3	*TET2, ATM, BCR, CLCN7, BRCA1, CBL, FANCA, PAX5, TBXAS1, NF1 and JAK2*
Filter 2.2 Variants related to MDS and AML MAF ≤ 0.05 Intronic were excluded	268	268	174	4	*TBXAS1, CUX1, FANCA, RTEL1, SAMD9L, SEC23B, TET2, ATM, BRCA1,*

					CTCFL, GP6, KMT2D, SLX4, XRCC4, ERCC6L2 and KLF1
Filter 3 Variants related to neutropenia eVai pathogenicity score ≥ 0.	195	71	173	4	*VPS13B, CSF3R, FUT8, MMAB, SLC35A1 and FANCA*
Filter 4 Variants related to bone marrow hypocellularity eVai pathogenicity score ≥ 0.	50	24	38	1	*PRF1, CLCN7, TERT, NHP2, VPS33A, DNAJC21, FANCI, LBR, TBXAS1 and WDR19*
Filter 5 Variants related to thrombocytopenia eVai pathogenicity score ≥ 0.	245	63	202	4	*TLDC2, MMAB, TNFRSF11A, and VWF*

Note: Only variants with high quality (QUAL ≥ 30) have been selected.

5.3 Phenotypic variation in two siblings affected with SDS: the use eVai suggests clinical relevance of a variant in the KMT2A gene

The major part of this study was published in Genes Journal on 23 of July 2022 (https://doi.org/10.3390/genes13081314). The full version of the paper is attached in the appendix section of the thesis.

In this study, we present two SDS siblings (UPN 42 and UPN43), both carrying the same *SBDS* pathogenic variants and showing relevant differences in their clinical presentation. By using filters 6 and 7 as described in methods chapter 3.4.3, we demonstrate that part of the clinical variability can be explained using WES and bioinformatics tools.

5.3.1 Case presentations

- ***UPN42***

She is a Caucasian female, her weight at birth was 2870g. In the first months of her life, she began to manifest a diarrheal alvus, and she was hospitalized several times for cough, fever and dyspeptic symptoms, with features of pancreatic insufficiency.

Since the first admission, a reduction in the number of neutrophils has been highlighted, and a hypothesis of autoimmune neutropenia was excluded. Gradually over time, the symptoms of diarrhea with the characteristics of steatorrhea became more severe (5-7 discharges/day). At the age of 1 year, the hypothesis of cystic fibrosis was excluded through the use of sweat test, and SDS was diagnosed; oral replacement therapy with pancreatic extract was undertaken with good results. In regular follow-up, up to the age of 21, the number of neutrophils was always lower than the average for the age. The hemoglobin values always remained within the normal range and also the count of reticulocytes was always appropriate to the hemoglobin levels. The platelet count was always lower than normal. Annual monitoring of bone marrow aspirates demonstrated poor or very poor cellularity, with notes of dyspoiesis in the myeloid precursors. The erythroid series in maturation was between 9 and 21% but it was not always observed; in the share of mature lymphocytes reached a minimum of 44% and a maximum of 63%. Megakaryocytes were almost always absent. No blasts were found in any bone marrow examination and no evidence of any clonal evolution.

She never requested blood transfusion or supportive therapy with G-CSF. At the age of 28, the FISH (Fluorescence In-Situ Hybridization) examination confirmed the presence of a cell clone in 90% of the nuclei examined, containing a deletion of the centromeric region of the chromosome 7 (7q11), consistent with the presence of i(7) (q10).

At the age of 32, she developed Hodgkin lymphoma, and recovered after standard therapies; follow-up is regular. The neuropsychological tests were normal, and the patient works as an employee.

- ***UPN43***

This subject is a Caucasian male, and the brother of UPN42. His weight at birth was 3450 g. An obstetric ultrasonography test revealed a horseshoe kidney with bilateral pyelectasis, more evident on the left kidney.

At the age of two months, features of seborrheic eczema with a significant atopic component were found, and at the age of 2.5 months, the patient developed chickenpox. At the same time, a growth delay was evident (4900 g, <3%; length 58 cm, <3%; head circumference 39.5 cm, <3%), and the appearance of greasy and foul-smelling stools was reported by his parents. Chemical-microscopic examination of the stool showed evidence of abundant neutral acids and fats and a tryptic power <2.5 UT; the sweat test was negative, and molecular confirmation of SDS was obtained. Weight recovery took place progressively, adapting the diet. At the age of 2 years, the hypothesis of pancreatic

insufficiency was confirmed, and oral replacement therapy with pancreatic extract and fat-soluble vitamin supplements was undertaken. Weight increase improved, and infectious episodes (bronchitis and persistent cough) were sporadic, similar to the episodes of steatorrhea.

At the age of 6 years, irregularities in the metaphyseal regions of the long bones, particularly at the level of the proximal and distal ends of the femurs and of the proximal ends of the tibias, were demonstrated. The metaphyseal regions appeared enlarged, frayed, and unevenly thickened. The femurs appeared curved with a medial concavity, and the coxo-femoral joints were regular. Slight metaphyseal irregularities were also appreciable in the humerus, radius and ulna. The ribs were short and squat. At the same age, he underwent orchidopexy due to cryptorchidism.

The hematological examinations consistently showed leukocyte counts low for his age. The hemoglobin values always remained in the normal range, while platelet counts remained low. Regular bone marrow monitoring, starting at the age of 15, always demonstrated poor cellularity with notes of dysmyelopoiesis. The erythroid series varied between 9% and 25%. The proportion of mature lymphocytes was always between 38 and 53%. Megakaryocytes have always been rare, and they were absent in the last check. No blasts have been found in any bone marrow examination, and no clonal evolution has been recorded.

Similar to his sister, he never requested blood transfusion or supportive therapy with G-CSF. FISH examination confirmed the presence of a cell clone in 90% of the nuclei examined, containing a deletion of the centromeric region of chromosome 7 (7q11), which is consistent with the presence of i(7)(q10).

At last, minor facial dysmorphic features such as hypertelorism and wide nasal bridge were recorded. Records of family history show that he always needed additional support at school and that he can be defined as having a mild developmental delay, with problems in the areas of expressive language and memory; he has obtained a job within a national support program for people with minor handicaps.

5.3.2 findings

For both patients, the clinical diagnosis of SDS had been previously confirmed by the demonstration of two pathogenic variants in the *SBDS* gene c. [258 + 533_459 + 403del] and c. [258 + 2T > C], inherited from the father and the mother, respectively.

Using eVai, and according to the filter used, we selected:

- **Filter (6):** 28 and 29 variants in UPN42 and UPN43, respectively (Tables 5.15 and 5.16)
- **Filter (7):** 14 variants in UPN42 and 82 variants in UPN43. The variants of filter 7 are attached at the end of the thesis as supplementary files (Tables .1.S and 2.S).

Table 5.15. *Variants found using Filter 6 for UPN42*

Gene	ACMG Classification	HGVS_Protein	HGVS_Coding	eVai Pathogenicity score	Zygosity	Effect
SBDS	Pathogenic	NA	c.258+2T>C	8.0	Het	splice_donor_variant
SPG11	Pathogenic	p.Met245fs	c.733_734delAT	8.0	Het	frameshift_variant
LIPA	Likely pathogenic	p.Gln298Gln	c.894G>A	6.0	Het	synonymous_variant
POU6F2	VUS	p.Gln212dup	c.635_637dupAGC	5.0	Het	disruptive_inframe_insertion
QRICH2	VUS	p.Arg969*	c.2905C>T	5.0	Het	stop_gained
CCDC40	VUS	p.Ala1028fs	c.3082dupG	5.0	Het	frameshift_variant
KRT74	VUS	NA	c.748-2A>G	4.5	Het	splice_acceptor_variant|
LAMB3	VUS	p.Gly508Arg	c.1522G>C	4.5	Het	missense_variant
SEC24D	VUS	p.Gly380Arg	c.1138G>A	4.0	Het	missense_variant
DM1-AS	VUS	NA	-	4.0	Het	intron_variant
ITGB4	VUS	p.Ala637Val	c.1910C>T	4.0	Het	missense_variant
TSC1	VUS	NA	c.2626-3delC	4.0	Het	splice_region_variant
CLCN4	VUS	p.Ile668Thr	c.2003T>C	3.5	Het	missense_variant
ATRX	VUS	p.Lys1185Asn	c.3555G>C	3.5	Het	missense_variant
ATP11C	VUS	p.Ile660Thr	c.1979T>C	3.0	Het	missense_variant
TEX11	VUS	NA	c.1004+265G>T	3.0	Het	intron_variant
EBF3	VUS	NA	c.555-20970G>A	3.0	Het	intron_variant
CLN3	VUS	p.Ala28Val	c.*69C>T	3.0	Het	3_prime_UTR_variant
TENM4	VUS	NA	c.494-592G>A	3.0	Het	intron_variant
EZH2	VUS	NA	c.363+70T>C	3.0	Het	intron_variant
CHST11	VUS	NA	c.204+57743G>A	3.0	Het	intron_variant
CHMP1A	VUS	NA	c.-114C>T	3.0	Het	5_prime_UTR_variant
C1QBP	VUS	NA	c.-80-956G>T	3.0	Het	intron_variant
AGBL1	VUS	NA	c.489-20094C>T	3.0	Het	intron_variant
RS1	Likely benign	p.Ile199Ile	c.597C>A	3.0	Het	synonymous_variant
ATP2A1	Likely benign	p.Asp951Glu	c.2853C>G	3.0	Het	missense_variant
GALC	Likely benign	p.Thr633Thr	c.1899G>T	3.0	Het	synonymous_variant
TNFRSF13B	Likely benign	p.Cys104Tyr	c.311G>A	3.0	Het	missense_variant

Table 5.16. *Variants found using Filter 6 for UPN43*

Gene	ACMG Classification	HGVS_Protein	HGVS_Coding	eVai Pathogenicity score	Zygosity	Effect
SBDS	Pathogenic	NA	c.258+2T>C	8.0	Het	splice_donor_variant
FLG	Pathogenic	p.Arg2447*	c.7339C>T	8.0	Het	stop_gained
TPRN	Pathogenic	p.Ser258fs	c.772_773insG	7.0	Het	frameshift_variant
KMT2A	Likely pathogenic	p.Gly3555Ser	c.10663G>A	5.5	Het	missense_variant
MSH3	VUS	p.Ala55_Pro63del	c.162_188delTGCAGCGGCCGCAGCGGCCGCAGCGCC	5.0	Het	disruptive_inframe_deletion
ROR2	VUS	p.Ile180Val	c.538A>G	4.0	Het	missense_variant
THBS2	VUS	p.Ala708Thr	c.2122G>A	4.0	Het	missense_variant
RNASEL	VUS	p.Glu265*	c.793G>T	4.0	Het	stop_gained
PLCB3	VUS	p.Ala10Val	c.29C>T	4.0	Het	missense_variant
PIP5K1C	VUS	p.Asp667Asn	c.1999G>A	4.0	Het	missense_variant
PMS2	VUS	NA	c.706-3delC	3.5	Het	splice_region_variant
BTD	VUS	p.Asp424His	c.1270G>C	3.5	Het	missense_variant
SLC5A2	VUS	p.His565Arg	c.1694A>G	3.5	Het	missense_variant
DM1-AS	VUS	NA	c.*281_*283delCTG	3.5	Het	intron_variant
TRPM6	VUS	p.Thr97Ala	c.289A>G	3.0	Het	missense_variant
KMT2C	VUS	NA	c.161+27108C>T	3.0	Het	intron_variant
KMT2C	VUS	NA	c.161+23318C>T	3.0	Het	intron_variant
MCM6	VUS	p.Ala93Asp	c.278C>A	3.0	Het	missense_variant
RASGRP1	VUS	NA	c.*500A>G	3.0	Het	3_prime_UTR_variant
LTBP3	VUS	p.Gly1234Gly	c.3702C>T	3.0	Het	synonymous_variant
SLC34A1	VUS	p.Val91_Ala97del	c.272_292delTCCCCAAGCTGCGCCAGGCTG	3.0	Het	disruptive_inframe_deletion
BCORL1	VUS	NA	c.*2C>T	3.0	Hom	3_prime_UTR_variant
GMPPB	VUS	NA	c.*227T>A	3.0	Het	3_prime_UTR_variant
RABEP1	VUS	p.Phe439Leu	c.1317T>G	3.0	Het	missense_variant
SLC7A14	VUS	NA	c.759+2212C>T	3.0	Het	intron_variant
LOC101929130	VUS	NA	c.-197G>A	3.0	Het	intron_variant
ATL1	VUS	NA	c.-139-11427C>T	3.0	Het	intron_variant
IL21R	VUS	p.Ser375Phe	c.1124C>T	3.0	Het	missense_variant

All the variants were evaluated singularly, as mentioned in the Material and methods section (3.5 variant interpretation). eVai, of course, identified *SBDS* variants as relevant for patients' phenotypes.

For UPN42, the single variant analysis did not disclose any variation, which could be related to the patient phenotype. Conversely, a *KMT2A* variant, (c.10663G>A) p.Gly3555Ser) was found to be related to some of the HPO terms that describe UPN43, including developmental delay, horseshoe kidney, bone abnormalities, cryptorchidism, expressive-language delay, and minor facial dysmorphisms of hypertelorism and a wide nasal bridge.

Pathogenic variants in the *KMT2A* gene cause Wiedemann–Steiner Syndrome (WDSTS, OMIM #605130), an autosomal dominant disorder. Interestingly, the phenotype of this syndrome includes all HPO terms selected for UPN43, which are absent in UPN42.
The variant was validated in the trio using Sanger sequencing and demonstrated to be *de novo* with a heterozygous status (Figure 5.24).

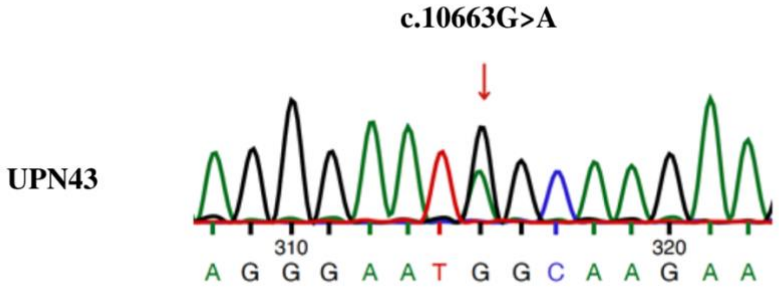


Figure 5.24. *Sanger sequencing confirmation. Part of the electropherogram illustrates the variant (c.10663G > A) in KMT2A in UPN43*

The *de novo* variant c.10663G > A in *KMT2A* is a novel one; it is unpublished, and no allele frequency is reported. The variant is classified as likely pathogenic according to the ACMG guidelines, and classified as damaging according to SIFT, MVP, FATHMM, Mutation Taster, M-CAP, and CADD. Uniprot alignment analysis showed that amino acid G (glycine) in position 3555 is highly conserved among different species such as humans, mice, rats, chickens, bovines, horses, and sheep (Figure 5.25). As a further confirmation, the digenic combination involving *KMT2A* and *SBDS* genes in the UPN43 sample was predicted as pathogenic by DIVAs and subclassified as a likely Dual Molecular Diagnosis.

Figure 5.25. *Uniprot alignment analysis shows that amino acid G (glycine) in position 3555 is highly conserved among different species such as humans, mice, rats, chickens, bovines, horses, and sheep.*

CHAPTER 6 – DISCUSSIONS

The discussion chapter is divided according to the three sub-studies that performed in this thesis.

6.1 Identification of variants in other genes related to SDS or SDS-like phenotypes

Three main features characterize SDS: bone marrow failure, pancreatic exocrine dysfunction and skeletal abnormalities (Dror et al., 2011). In addition, a significant percentage of SDS patients may develop serious haematological complications, requiring, in some cases, hematopoietic stem cell transplantation, a procedure associated with a requirements for specific protocols for this disease (Donadieu et al., 2012). A challenge for the surveillance of the disease is the early identification of risk factors relevant for the evolution of the haematological picture (Furutani et al., 2022). An extensive study on a French cohort of 102 patients postulated that the combination of a young age at diagnosis with low values of specific hematologic parameters, at the time of diagnosis and later, constitutes a negative prognostic factor. This combination was a risk factor stronger than any of the other 9 single variables taken into consideration alone, as transient cytopenia, low value of neutrophils, haemoglobin, platelets, or *SBDS* genotype. The latter was confirmed not to be linked to significative differences in hematologic parameters and other non-hematologic features, both in patients harbouring two common mutations or in patients with one common and one rare mutation (Donadieu et al., 2012).

Although the risk for developing MDS and AML is not related to the type of *SBDS* mutations, it can be favorably modified by other genetic factors as the presence of specific chromosomal abnormalities, in particular the i(7)(q10) and the del(20)(q), if they are the only abnormality present (Khan et al., 2021; Minelli et al., 2009).

When del(20)(q) is considered, cytogenetic studies, made over a long period of time on the SDS Italian cohort, confirmed the benign prognostic significance of this abnormality. Indeed, the patients carrying the del(20)(q) consistently showed a mild hypoplastic bone marrow picture, no or mild neutropenia, anaemia, and thrombocytopenia (Pressato et al., 2012; Valli et al., 2019b).

In all cases the del(20)(q) includes the loss of *EIF6* locus and, consequently, in the bone marrow clones with the chromosomal abnormality, only one allele expressing *EIF6* is present in the bone marrow clones with the chromosomal abnormality.

This condition has been studied *in vivo* on mice demonstrating that knockout heterozygous animals presented reduced eIF6 levels as expected, while preserving sufficient nucleolar eIF6 and regular ribosome biogenesis. In the liver cells a larger amount of 80S in polysomal profiles suggested a lower efficiency in initiation of translation (Gandin et al., 2008).

Common events in SDS patients are the development of many somatic clones in bone marrow with variants in either *EIF6* or *TP53* genes, producing different effects: i) variants in *EIF6* have a compensatory role on the impact of SBDS deficiency, in turn reducing the risk of developing MDS and AML by improving the ribosome maturation, enhancing translation, and reducing p53 upregulation (Kennedy et al., 2021), ii) variants in *TP53* act in a different pathway and decrease tumor suppressor checkpoints activation. Clones acquiring only variants in both *TP53* alleles are associated to the development of haematological malignancies (Kennedy et al., 2021).

Furthermore, higher p53 protein levels, linked to inhibition of osteogenesis, has been found is SDS patient's osteoblasts, in correlation with the skeletal anomalies of patients (Frattini et al., 2021).

Relevantly, the *EIF6* clonal variants in SDS patients were not observed in the presence of leukemic evolution or serious bone marrow dysfunction, confirming a positive impact on the haematological alterations determined by the *SBDS* genotype (Kennedy et al., 2021).

The results on humans fit the yeast model proposed by Menne et al. who demonstrated that missense variants in *Tif6* can bypass the fitness defect resulting from lack of *Sdo1* through the reduction of *Tif6* binding to the 60S ribosome subunit (Menne et al., 2007).

Our WES investigations detected a patient, UPN 2, in whom we found a heterozygous germline variant, c.100T>C (p.Phe34Leu), in EIF6. This variant is rare according to gnomAD and the amino acid Phe34 is extensively conserved (Fig 5.2). To the best of our knowledge, this is the first patients in whom a constitutional pathogenic *EIF6* variant is associated to biallelic *SBDS* mutations. In UPN 2, the missense variant in *EIF6* is a germline variant, thus at least in theory, its effect in ameliorating the consequences of *SBDS* mutations is expected to have started to act from zygote and to be widespread to all tissues.

Available clinical data suggest that stature (the patient is at low centiles for SDS patients), pancreatic function (the patient is still on therapy with pancreatic enzymes) and cognitive development are not strongly and favourably influenced. As far as the skeletal features are concerned, osteoblasts from UPN 2 did not differ with respect to osteoblasts from a group of 13 SDS patients studied by Frattini et al. (2021) who demonstrated lower mineralization capacity and lower expression of genes responsible for osteoblastogenesis (Frattini et al., 2021).

Conversely, haematological data and bone marrow picture, over the last ten years, have been stable: haemoglobin, platelets and lymphocytes have consistently been within normal limits and the patient never needed any kind of therapy. He has moderate cytopenia, which does not appear to have ever required transfusions or the use of hematopoietic growth factors.

Only mild bone marrow alterations where present and no evidence of any worsening has been reported so far, during the course of the disease. In addition, negative anti-p53 antibody reactivity has been repeatedly observed.

Similarly, Tan et al. (2021) reported an *SBDS*-mutated patient with a somatic bone marrow variant in *EIF6* (c.182G>T, p.Arg61Leu), showing stable and mild haematological features. While studying this patient, they interpreted the variant as an example of a somatic genetic rescue. The Arg61Leu variant in fact rescued the defect of Sdo1-deficient *yeast* cells and the larval lethality of Sbds deficient *Drosophila* (Tan et al., 2021). We expected that the variant p.Phe34Leu, identified in our patient, could have a similar effect as Arg61Leu, predicting a reduce binding to the nascent 60S ribosome, but it needs to be proven.

Our findings, on germinal level, might agree with the hypothesis that somatic *EIF6* mutations, observed by Kennedy and colleagues, produce a compensatory effect for the pre-existing germline *SBDS* mutations, thus reducing the risk to develop malignant neoplasia. Among of these somatic mutations, missense ones were predominantly located in regions encoding conserved secondary structure motives, exactly like the p.Phe34Leu variant described here, which is a newly described one (Kennedy et al., 2021).

In all SDS patients, the diagnosis prompts compulsory monitoring, over time, of the most relevant symptoms, including exocrine pancreatic dysfunction and, especially, bone marrow failure (Valli et al., 2017).

In our case, this program of surveillance will produce a large amount of useful information not only to manage the clinical problems of the patient but also to compare him with the group of the other SDS patients, whose exome analysis did not reveal any significant *EIF6* variants.

If the periodic clinical check will confirm a stable and positive situation, particularly concerning the haematological picture, we can confirm that we have observed a human example of somatic genetic rescue through *EIF6* mutations comparable to yeast and *Drosophila* models.

Undoubtedly, our observations, supported by genetic considerations and *in silico* simulations, may be of actual use for clinical interpretation only when experimentally validated.

In the future, the study of the effect of p.Phe34Leu variant could be also improved by using the molecular dynamics simulations approach as previously performed for EFL1 (Delre et al., 2020).

To check the presence, additional to *EIF6* variant, of any other variants in genes involved in MDS or AML (Lindsley, 2017; Steensma, 2017; Bluteau et al., 2018; Obrochta and Godley, 2018; Armstrong et al., 2018; Kennedy et al., 2021) we analysed the exome file of UPN 2 and three genes, *ASXL1*, *JAK2* and *GNAS*, were identified as bearing a mutation which might be relevant for the risk of MDS/AML (Table 5.2).

ASXL1: several studies showed that *ASXL1* somatic mutations are among the most frequent variants in all subtypes of myeloid malignancies including MDS (11-21% cases), AML (5-11%), myeloproliferative neoplasm (MPN) and chronic myelomonocytic leukaemia (CMML) (40-50%) (Boultwood et al., 2010; Fujino and Kitamura, 2020; Thol et al., 2011). *ASXL1* mutations accelerates myeloid malignancies and are always associated with poor prognosis (Gelsi-Boyer et al., 2012).

Confirmatory evidence of *ASXL1* relevance in hematological malignancies was produced by Wang and coworkers who showed that heterozygous genetic *ASXL1* knockout mice (Asxl1+/−) developed MDS/MPN (Wang et al., 2014), and by Abdel-Wahab and coworkers who reported that hematopoietic cell-specific deletions of Asxl1 induced a MDS-like disease (Abdel-Wahab et al., 2013). Germinal *de novo* nonsense or frameshift mutations in *ASXL1* cause the Bohring-Opitz Syndrome (Bedoukian et al., 2018), and a few reports describe missense germline mutations, in association with familial recurrence of different hematological malignancies.

Hamadou and coworkers reported the mutation p.Arg402Gln in two Tunisian sister affected by NHL (tonsil large B-cell lymphoma and gastric MALT lymphoma) (Hamadou et al., 2016); Seiter and coworkers reported father and son, both carrying the p.Asn968Ser mutation, who developed MDS then evolving in AML at age 75 and 46 respectively (Seiter et al., 2018).

Zebisch and coworkers described a family in which a 60-year-old female affected with MDS was transplanted from her 58 year-old apparently healthy sister; the graft function was poor, and laboratory investigations demonstrated the presence of the same *ASXL1* mutation, p.Pro808His, in the donor sister (Zebisch et al., 2020).

An *ASXL1* mutation, p.Gly704Arg, is present in a family (a 2-year-old child and her 54-year-old paternal uncle, both affected with primary myelofibrosis) included in the study of Andres-Zayas and coworkers on germline predisposition for leukemia (Andrés-Zayas et al., 2021).

In our patient, the missense variant p.Asp1211Asn is interpreted as pathogenic only by SIFT (https://sift.bii.a-star.edu.sg) (Vaser et al., 2016) (Table 5.2), and the previously reported germinal *ASXL1* mutations were reported as damaging or disease causing by SIFT and Mutation Taster respectively (https://www.mutationtaster.org) (Schwarz et al., 2014), while other tools classified them as benign.

Based on available evidence, missense germinal mutations in *ASXL1* might act as a risk factor for developing hematological malignancies; topics as penetrance or anticipation can be discussed only when a much larger number of cases have been studied (Seiter et al., 2018). At present hematological surveillance in carriers of germinal missense mutations in *ASXL1* seems a reasonable management of the problem, even if the functional significance of the mutation is not fully defined.

JAK2: the *JAK2* variant, p.Ser15Phe, is interpreted as pathogenic only in one out the seven prediction tools used (Table 5.2); it falls at the N-terminus outside the most functionally relevant domains reported by Benton and coworkers (Benton et al., 2019). Reports about germinal *JAK2* mutations are rare (Goldin et al., 2009; Park et al., 2020) and quoted mutations are described to be pathogenic when present in bone marrow as somatic mutations (Klampfl et al., 2013; Tefferi, 2016).

GNAS: the *GNAS* variant p.Glu717Ter, found in our patient and paternally inherited, is interpreted as pathogenic by all four applicable prediction tools (Table 5.2). Also, for *GNAS* reported evidence underlines its involvement in both genetic and hematological diseases.

Activating gain-of-function mutations are known to cause mendelian disorders as polyostotic fibrous dysplasia and McCune-Albright syndrome (Robinson et al., 2016). *GNAS* somatic mutations have been recurrently found in hematological disorders such as MDS and lymphoma (Bejar et al., 2011; Xie et al., 2014).

In addition, *GNAS* germline mutations are related to different type of Pseudohypoparathyroidism (PHP-Ia,-Ib,-Ic) and Pseudopseudohypoparathyroidism (PPHP). PHP and PPHP are autosomal dominant conditions and associated with lack of expression of Gsα if pathogenic variant inactivate maternal or paternal alleles respectively (Haldeman-Englert et al., 1993). As the clinical data of our patient includes increased PTH, a careful evaluation for endocrinological condition was suggested to the family and it is still in progress.

6.2 Distribution of germline variants related to the development of hematological abnormalities in 16 Patients affected with SDS

6.2.1 Hematological characteristics of patients

History of Neutropenia was found in all of our patients since it is essential for disease diagnosis. Thrombocytopenia and bone marrow hypocellularity were common and identified in 12 (75%) and 13 (81%) of patients, respectively. Other complications were less frequent, MDS and aplastic anemia were found in one patient (UPN 45), anemia (in UPN 24), Hodgkin lymphoma (in UPN 42), leukopenia and decreased circulating antibody levels (in UPN85).

Using the granulocyte colony-stimulating factor (G-CSF) and other neutropenia treatments, neutrophil count, in some cases, got improved over time, explaining their current normal values. The incidence of severe neutropenia (280-750 cell/L) in our group was estimated to be 25% (4/16 patients). Differently, the incidence of severe neutropenia was estimated to be 40% (10/25 patients) by Thompson and coworkers (Thompson et al., 2022), and 59.9% in the Italian SDS Registry study of 121 patients (Cesaro et al., 2020). On the other hand, compared to the findings of Thompson et al (25%), and Cesaro et al (66.8%), the incidence of thrombocytopenia in our study was higher and assessed to be 75%.

In the French national cohort study of 102 SDS patients, anemia was observed in 7 cases (6.25%) (Donadieu et al., 2012). Similarly, anemia was estimated in 6.25% of our patients. Myeloid malignancy of MDS was identified in 6.25% of our patients.

However, the incidence of myeloid malignancy was reported to be 9.8% by Cesaro et al. and 17% by Furutani et al.

Hodgkin lymphoma (HL) was diagnosed in one patient; this condition has never been reported in any SDS patients (Taha et al., 2022). One of our patients was diagnosed with leukopenia, a rare condition reported in some SDS patients (Alves et al., 2013).

6.2.2 Distribution of germline variants related to the development of hematological malignancies, including MDS and AML

The term MDS refers to a wide spectrum of myeloid neoplasms which share the characteristics of bone marrow failure, abnormal cell morphology, and a high predisposition for AML (Hellström-Lindberg et al., 2020). It is now well understood that MDS and AML, like other malignancies, are influenced by recursive rounds of positive selection, with gene mutations and other genetic abnormalities playing a significant role (Genovese et al., 2014).

In the WHO 2016 classification, myeloid neoplasms with germline predisposition were recognized as a distinct category. Germline predisposition increases the likelihood of myeloid neoplasms, primarily AML and MDS. According to studies, 5–15% of MDS or AML patients have germline pathogenic variants (Churpek, 2017).

The past ten years have seen a dramatic improvement in our understanding of the genetics of MDS and other myeloid illnesses by revolutionized sequencing technologies and new bioinformatic techniques that played a major role. A comprehensive registry of driver mutations recurrently found in a noticeable fraction of MDS patients has been revealed through intensive efforts of sequencing a large number of MDS genomes. Ongoing efforts are being made to clarify their effects on clinical phenotype and prognosis as well as their role in the pathogenesis of MDS (Ogawa, 2019).

In this study, using eVai, a new technology based on artificial intelligence, we filtered out and prioritized variants in genes related to the development of MDS and AML to determine the distribution of germline variants and to understand the genetic heterogeneity among our SDS patients.

The distribution of gene mutations was investigated in different subtypes of variant filtration. However, our protocol relied on pathogenicity scores, allele frequencies, and variant effects.

In filter 2.1, we selected all variants with a pathogenicity score ≥ 0. Overall, variations in 87 genes were detected, and *TET2, ATM, BCR, CLCN7, BRCA1, CBL, FANCA, PAX5, TBXAS1, NF1* and *JAK2* genes were the most commonly mutated genes among our SDS cohort.

All of these frequently mutated genes were observed in several patients affected with different types of hematological malignancies, either at the somatic or germline levels (Gilad et al., 2022; Maifrede et al., 2021). We also found that UPN45 (the only patient who developed MDS) had the highest number of variations.

Then, filter 2.2 was used to select variants with MAF ≤ 0.05 and coding (delins, missense, start loss, stop gained, and splicing). As a result, we found 268 genetic variations. Interestingly, UPN 45 was found to be significantly carrying the highest number of variants, in line with the results of filter 2.1. The most commonly mutated genes were found to be *TBXAS1, CUX1, FANCA, RTEL1, SAMD9L, SEC23B, TET2, ATM, BRCA1, CTCFL, GP6, KMT2D, SLX4, XRCC4, ERCC6L2*, and *KLF1* (Figure 5.7).

Only two variants in the *BRCA2* (c.7825G>A), and *SEC23B* (c.40C>T) genes were classified as pathogenic and found in UPN45, and UPN24, respectively. Two variants were classified as likely pathogenic in the *KMT2A* (c.5336T>G, and c.10663G>A) and *SEC23B* (c.1781T>G) have been also identified in UPN 45 and UPN43.

Due to higher vulnerability to double-stranded DNA breaks, the homologous DNA repair pathway gene *BRCA2* is classically linked to increased susceptibility to hereditary breast and ovarian cancer (mutator phenotype) (Evers et al., 2010). Mutations in this gene could also increase the risk of MDS and AML (Williams et al., 2018; Yang et al., 2022). For instance, in large populations of patients with breast cancer, those with hereditary homologous repair (HR) abnormalities had a higher risk of developing therapy-related MDS and AML. However, the exact relationship between *BRCA2* gene mutations and spontaneous myeloid neoplasms has yet to be elucidated. Moreover, the numerous clinically uncharacterized single nucleotide variations (SNV) at *BRCA2* gene loci complicate the analysis. Recent studies showed *BRCA2* and *BRCA1* are significantly overrepresented in the MDS/AML cohort as compared to the general population (Williams et al., 2018). However, the variants (c.7825G>A, c.4258G>T and c.5744C>T) found in the *BRCA2* have never been reported before (Pubmed, Google Scholar, ClinVar, Varsome, November 2022).

SEC23B is a key component of the coat protein complex II (COPII)-coated vesicles that transfer secretory proteins from the endoplasmic reticulum (ER) to the Golgi complex.

Germline mutations in the *SEC23B* are associated with both Cowden syndrome (CS) and congenital dyserythropoietic anemia type II. CS is an autosomal dominant condition characterized by multiple noncancerous, tumour-like growths called hamartomas and an increased risk of developing certain cancers including thyroid, breast, skin and endometrial. (OMIM:616858).

The likely pathogenic variant (c.1781T>G) in the *SEC23B*, found in UPN43, was reported in a group of cases affected with Cowden syndrome.

To date, no evidence of any hematological malignancies in the CS has been observed (Pubmed, Google Scholar, ClinVar, Varsome, November 2022).

CDAs is a rare autosomal recessive hematogical disorder characterized by hemolysis, ineffective erythropoiesis, and erythroblast morphologic abnormality in the bone marrow (OMIM: 224100) (Aydin Koker et al., 2018). However, the variant c.40C>T in the *SEC23B* was found in some patients with CDA type II. It is worth mentioning here that the MAF of c.40C>T was found to be significantly higher in Italy compared to the rest of Europe (26.3% vs. 10.7%) (Russo et al., 2011).

Germline mutations in *KMT2A* cause autosomal-dominant Wiedemann–Steiner Syndrome (WDSTS, OMIM #605130), and, in the vast majority of cases, *de novo* mutations have been confirmed (Luo et al., 2021). Since the initial association between WDSTS and *KMT2A*, more than 250 sequence variations have been described (Jones et al., 2012). *KMT2A* likely pathogenic variant (c.10663G>A) found in UPN 43 have been described in detail in our recent paper that was published as a part of this Ph.D. project (Taha et al., 2022).

KMT2A somatic mutations are common in leukemias of various lineages. Particularly, 3% of adult AML patients and 5% to 15% of adolescents and young adults with acute lymphoblastic leukaemia have *KMT2A* chromosomal rearrangements (ALL)(Bataller et al., 2021). Until now, no patients with WDSTS were reported to develop any type of hematological malignancy, so there is no evidence that the variants we found act as risk factors for UPN43 and UPN45.

Additionally, using the PathCards database, we evaluated the genetic pathways for the most frequently mutated genes. We found that 34% of the genes belong to the DNA repair pathway, 16% of genes play roles in signalling, 16% in transcriptional regulation and 16% in BRCA1 pathways, 12% in the metabolism pathway and 6% as a tumor suppressor (Figure 5.11).

Except for UPN 2, we found that all patients are carrying variants in genes involved in the DNA repair pathway. Among all of the patients, only UPN45 is carrying variants in all selected pathways. Except for UPN 2, all patients had variants in genes related to the DNA repair pathway. It is also interesting that only UPN 45 was found to be harbouring variants in all the genetic pathways that were found (Figure 5.12).

Genes involved in DNA methylation, chromatin remodeling, RNA splicing, transcription, signal transduction, cohesin regulation, and DNA repair are among the key mutational targets in MDS.

These mutations are thought to have a similar clonal origin since they exhibit significant overlaps with driver mutations seen in AML and age-related clonal hematopoiesis in healthy persons (Kontandreopoulou et al., 2022). It is believed that mutations are acquired and positively selected in a well-organized manner to let the development of the initial clone compromise normal hematopoiesis. This compromise leads to the development of MDS, which in many patients later transforms into AML. Significant correlations between mutations suggest the presence of functional interactions between mutations, which dictate disease progression (Ogawa, 2019).

Based on the aforementioned, we postulate that, in addition to the pre-existence of *SBDS* mutations, the higher number of variations found in UPN 45 as a result of filters 2.1 and 2.2 as well as the presence of variants in various genetic pathways, could all play a role in the development of MDS in the patient.

Since mutations are frequently linked to particular disease phenotypes, medication responses, and clinical outcomes, understanding MDS genetics is crucial for improving patient care (Ogawa, 2019).

The development of surveillance programs in addition to the identification of a genetic predisposition to MDS in patients with SDS may open the door to future clinical surveillance and management before the onset of severe disease. Early detection provides the chance for a prompt bone marrow transplant before the onset of leukemia because AML has a particularly poor prognosis, especially for patients with SDS (Kennedy and Shimamura, 2019).

However, It should be noted here that, for MDS and AML, personalized medicine, and the development of novel therapeutic compounds with the potential to significantly improve patient outcomes are still lagging behind that of many other blood malignancies (Hellström-Lindberg et al., 2020).

6.2.3 Distribution of germline variants related to the development of neutropenia

Neutropenia, typically defined as an absolute neutrophil count <1500/μL, is a common reason for referral to both adult and pediatric haematologists. although the majority of neutropenia episodes are temporary and external to the bone marrow, the identification of underlying hereditary neutropenia significantly alters clinical management (Donadieu et al., 2013). Consideration of an inherited syndrome should be given to patients who have a personal or family history of MDS or AML, persistent symptomatic or severe neutropenia (ANC 500 cells/L) without an obvious cause (Bejjani et al., 2017).

However, many of the genetic neutropenia conditions are linked with leukemia predisposition, or additional medical co-morbidities like SDS (Furutani et al., 2019). Persistent or intermittent neutropenia is a frequent presenting finding in almost all affected children, often before the diagnosis of SDS is obtained (Valli et al., 2017).

In this study, using eVai, we filtered out all variants with a pathogenicity score ≥ 0 in genes related to neutropenia using the HPO number (HP:0001875) to determine the distribution of these variants (filter 3).

As an outcome, we identified a total of 195 genetic variations in 54 genes, most of them were intronic (63.5%) and classified as VUS (89%), and only 1.3% were classified as pathogenic or likely pathogenic. The average number of variants per patient is 48, with an SD of 8.3, (range, 33- 62).

The highest number of variants were found in UPN 24, UPN 57 and UPN 20. All of those patients were grouped in group 1.1 (patients with severe neutropenia). On the other hand, UPNs 45, 15, 64 and 86 carried the lowest number of variants. Those cases were grouped into groups 1.2 and 1.3 (patients with moderate and normal neutropenia). Interestingly, Pearson correlation testing showed a negative correlation between the absolute neutrophil count and the number of variants identified in all patients (R=-0.601, p=0.05). According to our results, we presume that is more likely to have a neutrophil count lowered if the patient has a higher number of neutropenia-related variants. These novel findings, especially if it confirmed in other studies with larger sample sizes in the future, can be utilized in genomic surveillance and personalized medicine for SDS.

We observed that the genes *VPS13B* (found in 68.75% of cases) and *CSF3R* (62.5%) were the most frequently mutated, and their variants were found to be more common among patients in groups 1.1 and 1.2 of severe and moderate neutropenia (Table 5.15). In addition, variants in four genes (*FUT8, MMAB, SLC35A1* and *FANCA*) were identified in 37.5% of patients.

Pathogenic variants in the vacuolar protein sorting 13 homolog B (*VPS13B*, also referred to as *COH1*) gene, located on chromosome 8q22.2, cause Cohen syndrome (OMIM #216550), an autosomal recessive disorder that affects many parts of the body and is characterized by intellectual impairment, small head size (microcephaly), growth retardation, hypotonia and neutropenia (Rodrigues et al., 2018). In this study, a total of 14 genetic variants in the *VPS13B* gene were inherited in a heterozygous manner. There were only three missense variants found (c.7678G>A, c.1768G>A, and c.2518G>A), and all the other variants were intronic.

However, the two missense variants (c.2518G>A and c.7678G>A) were detected in UPN 24 (case from group 1.1). Although it should be mentioned that at the time of writing, parental status is unknown, the patient did not show any notable clinical relevance to Cohen syndrome.

CSF3R is a receptor for colony-stimulating factor 3 and is thought to play a prominent role in the growth and differentiation of granulocytes. Homozygous or compound heterozygous *CSF3R* mutations have been detected with a frequency of more than 30% in severe congenital neutropenia (CN) patients (OMIM, 617014, AR). *CSF3R* mutations are found in more than 80% of congenital neutropenia patients who develop AML or MDS, suggesting that they could play a significant role in leukemogenesis (Klimiankou et al., 2016).

On the other hand, chronic neutrophilic leukemia (CNL) has been associated with heterozygous mutations in the same gene (OMIM,162830, AD) (Duployez et al., 2019). In 2013, the majority of patients with CNL were shown to have recurrent mutations in the CSF3R gene, which revolutionized our knowledge of the disease's aetiology and established a biomarker for CNL diagnosis (Maxson et al., 2013).

In 10 cases, we identified six missense variants in the *CSF3R* gene (Table 5.7). With an eVai pathogenicity score of 4.5, the variant (c.1853C>T) was classified as pathogenic, while the other five variants listed in table 6.5 were classified as VUS. MAF for all variants was found to be less than 0.01%. Overall, among all neutropenia-related genes, *CSF3R* variants had the lowest MAF mean and the highest mean pathogenicity scores (Figures 5.18 and 5.19). Interestingly, we observed that three out of four patients (UPNs 58, 57 and 20) with severe neutropenia carried biallelic variants in the *CSF3R* gene. On the other hand, UPNs 45, 43, 85, 68, 51 and 64 were found to be harboring heterozygous variants.

In the present study, we noticed the presence of other variants that are likely to have an additional effect, especially in patients with severe neutropenia. In UPN 58, the patients with the lowest ANC (250 cells/L), we identified, in addition to the *CSF3R* mutations, a rare variant (c.107G>C) that was classified as VUS in the growth factor independent-1 (*GFI1*). Mutations in the *GFI1* have previously been found in patients with severe congenital neutropenia 2, an autosomal dominant condition (Muench et al., 2017). In UPN24, a heterozygous missense variant (c.1162C>G) in the *MDM4* has been identified. The variant is classified as VUS with a MAF of 0.0006. The *MDM4* gene encodes a protein that, along with *MDM2*, acts as a negative regulator of transcription factor *TP53*. The germline missense mutations of *MDM4* have been found in some cases with features suggestive of ribosomopathy dyskeratosis congenita (DC), neutropenia, bone marrow hypocellularity, short telomeres, and AML (Toufektchan et al., 2020). In UPN 20 and 57, we observed the presence of variants in the 40S ribosomal protein S19 (RPS19) and the 40S ribosomal protein S7 (RPS7). Both variants were classified as VUS with very low frequency (MAF ≤ 0.05). The *RPS7* and *RPS19* mutations have been linked to the ribosomopathy Diamond-Blackfan anemia (DBA). Ribosomopathies have an exceptionally wide range of phenotypes (Heijnen et al., 2014). However, many ribosomopathies, like SDS, DC and DBA have some features in common including cytopenia and growth defects. Therefore, in addition to the effect of the pre-existence of *SBDS* on ribosome stress, we postulate that the presence of variants related to other ribosomopathies like *MDM4*, *RPS19* and *RPS7* could have an additional influence on the patient's phenotypes like neutropenia.

The utilization of new techniques based on artificial intelligence to analyze WES by using the HPO term of neutropenia as main filter led to improving our understanding of the genetic basis in particular for severe or moderate neutropenic patients with the known causative gene of *SBDS*. Using this method, we identified new genes that either were observed as commonly mutated (*VPS13B* and *CSF3R)* or found in single-case observations (*GFI1, MDM4, RPS7* and *RPS19*). Certainly, future study of these genes and their relationship to *SBDS* would be recommended.

6.2.4 Distribution of germline variants related to bone marrow hypocellularity

Bone marrow hypocellularity, which can occur when there is decreased production in one or more hematopoietic cell lineages (myeloid or erythroid), is a common feature in SDS. Studies showed that 50-73% of SDS patients had hypocellularity in their bone marrow at the first biopsy of diagnosis (Thompson et al., 2022).

In our study, the patients were grouped according to their bone marrow status into severe (3 patients), moderate (5 patients) and normal (8 patients).

Filter 4 was performed using variants in genes related to the HPO term of bone marrow hypocellularity. As a result, we obtained a total of 50 genetic variations in 16 genes. Approximately, 50% of variants were exonic. Only one variant in the *PRF1* gene was inherited in homozygous status. One variant in the *LBR* gene was classified as pathogenic, 11 variants (13%) were classified as likely benign while and the vast majority of variants (N=38, 76%) were classified as VUS.

We found that nine patients were harboring variants in the *CLCN7* and *PRF1* genes, four patients had mutations in *TERT, NHP2*, and *VPS33A*, while three cases had variants in *DNAJC21, FANCI, LBR, TBXAS1*, and *WDR19*. Unlike neutropenia, we did not find any correlation between the number of variants that resulted from filter 3 and the bone marrow status.

All of the chloride channel 7 (*CLCN7*) variations that were found were intronic, classified as VUS, and inherited in a heterozygous state. The mutations of the CLCN7 gene, which impairs bone resorption, are the primary cause of autosomal-dominant osteopetrosis type II (ADOII) (Song et al., 2022).

On the other hand, the *PRF1* gene is located on chromosome 10q22.1 and encodes the perforin protein which is responsible for lymphocyte granule-mediated cytotoxicity. Target cells cannot be killed by the immune system in patients with perforin deficiency because the released cytotoxic granule contents cannot penetrate these cells (Canna and Marsh, 2020). Mutations in the *PRF1* gene have been associated with hemophagocytic lymphohistiocytosis (HLH), AR (OMIM: 603553), non-Hodgkin lymphoma (OMIM:605027) and aplastic anemia (OMIM:609135). HLH is characterized by the phagocytosis of various cells by histiocytes in the bone marrow. Additional features of hypocellularity of bone marrow have been found in some cases (Chandra et al., 2014).

Pancytopenia, coagulation disorders, hypofibrinogenemia, and hypertriglyceridemia have all been detected in laboratory tests. Ataxia, neurological dysfunction, and seizures are frequent (OMIM:603553).

We identified three missense variants (c.272C>T, c.755A>G and c.632C>T) in the *PRF1* gene. All the variants have been reported in previous studies. A meta-analysis study found a significant relationship between the homogenous or complicated heterogenous c.272C>T variant and the risk of HLH (Zhu et al., 2020). Interestingly, biallelic variants in the *PRF1* have been identified in 2 out of 3 patients with severe bone marrow hypocellularity either in homozygous (UPN58) or compound heterozygous status (UPN45) (Table 5.11).

Studies suggested that c.755A>G and possibly other *PRF1* variations are susceptibility factors for type 1 diabetes development (Orilieri et al., 2008). Importantly, this variant has been found in UPN 45 who has been diagnosed with type 1 diabetes (Table 4.2).

Additionally, the monoallelic variant (c.632C>T), found in UPN42, has been subsequently described in some cases affected with Hodgkin lymphoma (HL) (Ciambotti et al., 2014). Noted that, UPN42 has been diagnosed with HL 3 years ago.

However, according to El Abed et al, it is likely that the heterozygous *PRF1* variant could act as an inherited risk factor combined with other genetic variations (somatic or constitutional alterations in a second allele) and/or in the presence of environmental factors (El Abed et al., 2011).

Finally, future research should focus on investigating the precise function of *PRF1* and other genes in SDS patients with severe bone marrow hypocellularity using greater samples. For SDS individuals with severe bone marrow pictures, the identification of predisposing genetic markers would assist in the development of genetic counseling and testing programs. These cases with high risk can be identified using molecular screening protocols.

6.2.5 Distribution of germline variants related to thrombocytopenia

Thrombocytopenia, defined as a platelet count of less than 150,000 mcL, was found in 12 of our cases. Five patients (UPNs 24, 6, 20, 43 and 85) were classified into group one (patients with severe platelet count reduction of less than 100,000 mcL).

By using filter 5, we obtained 245 genetic variations in 66 genes. Only 63 variants were missenses and 8 were inherited in homozygous status. Additionally, we identified four likely pathogenic variants in *CD36* (c.949dupA), *VWF* (c.2561G>A), *ADAMTS13* (c.577C>T) and *SRC* (c.1579G>A) genes.

Unlike neutropenia, but similar to bone marrow hypocellularity, using the Pearson correlation test, no statistically significant correlation between the number of variants and platelet counts has been found (r= -0.142, *P*=0.599).

The genes *TLDC2, MMAB, TNFRSF11A*, and *VWF* were the most frequently mutated. Six cases harbor the *MMAB, TNFRSF11A*, and *VWF* variants, whereas nine cases have the TLDC2 variations. However, all the variants identified in the *TLDC2* (N=5), *MMAB* (N=6), and *TNFRSF11A* (N=4) are intronic and have MAF >0.05.

On the other hand, regarding the Von Willebrand factor (*VWF*) gene, 3 missense variants (c.7636A>T, c.2561G>A and c.3332G>A) have been identified. Interestingly, *VWF* variants have been noticed only in patients with severe thrombocytopenia. The variant c.2561G>A, found in UPN6, was classified as likely pathogenic with an eVai pathogenicity score of 5.5, whereas the variants c.7636A>T and c.3332G>A were classified as VUS and found in UPN 85 and UPN 20, respectively.

The VWF is a significant modulator of platelet-vessel wall interaction and platelet adhesion as well as a carrier for coagulation factor VIII. Von Willebrand disease types 1 and 3 as well as 2A, 2B, 2M, and 2N have been associated with heterozygous mutations in the VWF gene (OMIM:613160).

The single observations of patients with severe thrombocytopenia have indicated the presence of genetic variants with high pathogenicity scores; in UPN 24, the patient with the lowest platelets count (71000 mcL), was found to be having the rare variant c.577C>T in the *ADAMTS13* gene. This variant was classified as likely pathogenic with an eVai pathogenicity score of 6.

The ADAMTS13 is a multidomain protease that cleaves VWF in circulating blood and thereby limits platelet thrombosis. Alternatively, *ADAMTS13* is called Von Willebrand factor-cleaving protease; VWFCP. Mutations in this gene can cause hereditary thrombotic thrombocytopenic purpura (TTP) with autosomal recessive inheritance (OMIM: 604134). Moreover, the same patient also is carrying another likely pathogenic variant (c.1579G>A) in the tyrosine kinase *SRC* gene. This variant, in heterozygous status, has been previously described in some patients with thrombocytopenia, myelofibrosis, bleeding, bone diseases, premature edentulism, and minor facial dysmorphia. These patients have fewer and heterogeneous-sized platelets that are hyporeactive to collagen. Functional studies revealed that the variant c.1579G>A makes the *SRC* incapable of its self-inhibition, leading to constitutively active SRC expression in platelets and causing bleeding to inhibit collagen-induced platelet activation while stimulating platelet spreading (De Kock and Freson, 2020; L et al., 2019).

In UPN85, in addition to the variant c.7682T>A in the *VWF* gene, the patient was also found to be harboring a compound heterozygous rare variants (c.1208T>C, c.1233C>A) in the *SLC46A1* gene. Both variants were classified as VUS with an eVai pathogenicity score of 2.5. Homozygous or compound heterozygous mutations in the *SLC46A1* gene are associated with hereditary folate malabsorption (HFM), (OMIM: 611672). Features of anemia, thrombocytopenia, neutropenia and leukopenia are frequently observed in patients affected with HFM (Zhao et al., 2017).

Here, we postulate that the presence of variants with high pathogenicity scores in *VWF*, *ADAMTS13*, *SRC* and *SLC46A1* genes might have an additional effect on patients who presented with severe platelet count reduction.

6.3 Phenotypic variation in two siblings affected with SDS: the use eVai suggests clinical relevance of a variant in the KMT2A gene

In this study, we presented two SDS siblings, (UPN42, female, and UPN43, male), who shared classical SDS features such as pancreatic insufficiency, hematological abnormalities (neutropenia, thrombocytopenia), and bone marrow hypocellularity. UPN43 showed additional characteristics, not found in his sister, including skeletal problems (metaphyseal irregularities, metaphyseal regions enlargement, curved femurs, short ribs, and varus cervico-diaphyseal), renal abnormalities (horseshoe kidney with bilateral pyelectasis), recurrent infections, cryptorchidism, developmental delay, and minor dysmorphisms.

As an attempt to understand the clinical variability found in the two siblings, we analyzed WES data using the eVai and DIVAS platforms.

In UPN42, Hodgkin lymphoma (HL) was diagnosed; this disease was never reported in any SDS patients, and the use of the eVai platform and DIVAs failed to identify any relevant variation, in keeping with the evidence of minor relevance of genetic factors in the development of HL. Recently, Weniger and a co-worker listed a number of single nucleotide variants found to be associated with HL in a limited number of cases. However, none of the variants listed were found in UPN42 (Weniger and Küppers, 2021).

In UPN43, in addition to *SBDS* mutations, we identified and validated a heterozygous *de novo* missense variant located in exon 27 of the *KMT2A* gene (Figure 5.24). The variant is classified as likely pathogenic according to the ACMG guidelines, its pathogenicity score of eVai is 5, which is considered a high score.

As the variant (c.10663G>A, p. Gly3555Ser) in *KMT2A* was not reported before, no information related to its frequency is available; two pathogenic have been identified close to the site where our variant was found, within 100 bp (Baer et al., 2018).

KMT2A is located on chr11q23.3, consists of 37 exons, and encodes a DNA-binding protein that methylates a lysine residue on histone H3 (H3K4) . According to mice studies, *KMT2A* is abundantly expressed in adult hippocampus neurons and is required for synaptic plasticity, cognition, complex behaviors, and long-term memory (Jakovcevski et al., 2015; Kim et al., 2007). Pathogenic variants in the *KMT2A* cause chromatin remodeling deficiencies, which lead to widespread alterations in gene expression throughout development, resulting in abnormalities in multiple body systems (Foroutan et al., 2022). The reported variants are located quite uniformly through the sequence of the gene and most variants are clustered in exon 27, which is compatible with the fact that it is the longest exon (Baer et al., 2018; Fontana et al., 2020).

Germline mutations in *KMT2A* cause the autosomal dominant Wiedemann-Steiner Syndrome (WDSTS, OMIM #605130), and, in the vast majority of cases, *de novo* mutations have been confirmed (Sheppard et al., 2021). Since the initial association between WDSTS and *KMT2A* (Jones et al., 2012), more than 250 sequence variations have been described (Fontana et al., 2020a; Jones et al., 2012).

WDSTS is an exceptionally rare, <1/1000,000, chromatinopathy disorder characterized mainly by distinctive facial dysmorphism, hypertrichosis cubiti, developmental delay, skeletal anomalies, short stature, psychomotor delay, horseshoe kidney, ocular, cardiac, and dental manifestations (Baer et al., 2018; Grangeia et al., 2020a; Stellacci et al., 2016). The phenotypic spectrum of the disease is very wide, and extensive clinical variability has been reported (Jones et al., 2012; Sheppard et al., 2021), and further expanded finding more cases by WGS and WES (Giangiobbe et al., 2020). The WDSTS genotype-phenotype correlation is currently not fully understood, and the mild/unusual WDSTS presentations may be challenging to be recognized (Squeo et al., 2020).

Intellectual disability/ psychomotor delay, usually mild to moderate, was reported in 65-100% of cases. The prevalence of Autism has been estimated to be 11.8%, but subjects without autism may also reveal behavioral abnormalities such as ADHD, anxiety, and emotional dysregulation (Min Ko et al., 2017).

Facial dysmorphisms are common (50-70%), and differ from one patient to another, including hypertelorism, long and downslanting palpebral fissures, long eyelashes, wide nasal bridge, low-set ears, thin vermilion, micrognathia, and anomalies of the dentition (Strom et al., 2014; Sun et al., 2017).

Skeletal abnormalities such as hip dysplasia, delayed bone maturation, short palm, fifth finger clinodactyly, and small and buffy hands have been found in about half of patients. Hypertrichosis cubiti was approximately reported in about 60% of cases (Aggarwal et al., 2017a; Baer et al., 2018).

The wide clinical spectrum also includes a significantly higher risk of developing recurrent infections (Bogaert et al., 2017). Congenital heart disorders (in 30% of cases and including septal defect, and patent ductus arteriosus). Ocular manifestations (such as ptosis, squint, lacrimal duct anomalies, and refractive errors, in about 50% of cases) (Li et al., 2018). Renal anomalies including horseshoe kidney, pyelectasis, small or hypoplastic kidneys, and cryptorchidism have been reported in about 30% of cases (Baer et al., 2018; Fontana et al., 2020b; Grangeia et al., 2020b; Lebrun et al., 2018).

If we compare the phenotype of UPN43 with classical SDS description and WDSTS, we note that hematological problems and pancreatic insufficiency are SDS related and not reported in WDSTS. Developmental delay, of variable severity, is found in about 65-76% of SDS cases (Perobelli et al., 2012), so it is part of the typical description of the syndrome. It is also reported in 65-100% of WDSTS patients (Aggarwal et al., 2017b; Sheppard et al., 2021). At present, as the percentages of patients with developmental delay are roughly similar in both syndromes, we cannot assign or define the specific contribution of each gene to this problem.

Although skeletal abnormalities are reported in about 50% of patients with WDSTS, the skeletal features of metaphyseal irregularities, curved femurs, and short ribs, present in UPN43, are considered classical for patients with *SBDS* mutations and more compatible with SDS rather than WDSTS. In the latter syndrome, in fact, the typical skeletal abnormalities are hip dysplasia, delayed bone maturation, short palm and fifth finger clinodactyly (Aggarwal et al., 2017b; Baer et al., 2018).

The recurrent infection episodes, present in UPN43, are typical for SDS as a consequence of neutropenia, which is mild to moderate, on different occasions. Infections have been reported in both syndromes, and we have no evidence, at present, of any interaction between *SBDS* and *KMT2A* which could be related to the worsening of infections.

Conversely, horseshoe kidney with/ without pyelectasis was never reported in any SDS patient (PubMed and google scholar search, June 2022, using horseshoe kidney AND Shwachman-Diamond Syndrome), is found in approximately 0.25% of the general population, and more commonly reported in WDSTS with 6 patients showing this malformation (Aggarwal et al., 2017b; Baer et al., 2018; Castiglioni et al., 2022; Grangeia et al., 2020b; Nardello et al., 2021).

Similarly, cryptorchidism (not reported in patients with *SBDS* mutations, PubMed and google scholar search, June 2022, using cryptorchidism AND Shwachman-Diamond Syndrome) is present in UPN 43, its prevalence in the normal population is 1.7%, and observed in about 20-35% of WDSTS cases (Demir et al., 2021; Grangeia et al., 2020b; Nardello et al., 2021). However, despite that cryptorchidism has been reported in some WDSTS patients, it has never been associated with *KMT2A* mutations, instead, it has been associated with *TASP1*, which is a regulator of *KMT2A* (Suleiman et al., 2019).

Features of facial dysmorphisms, horseshoe kidneys, pyelectasis, and cryptorchidism are not hallmarks of SDS, but instead, they could be more likely related to WDSTS. Therefore, we postulate that the *KMT2A* variant, found in UPN43, for which there is *in silico* evidence of being pathogenic, has a concomitant and co-occurring clinical effect. This dual molecular effect, supported by in silico prediction with the DIVAs tool, could likely help to understand some of the clinical variations found among the siblings.

KMT2A was previously known as MLL (Mixed Lineage Leukemia) because of its recognition as a frequent target of somatic rearrangements in acute leukemia (Peterson et al., 2018). Up to now, no patients with WDSTS were reported to develop any type of hematological malignancy, so there is no evidence that the mutation we found may act as a risk factor for UPN43.

CHAPTER 6 – CONCLUSIONS AND FUTURE PROSPECTIVES

In the first sub-study of the thesis, UPN2 was diagnosed with SDS by targeted mutation analysis. WES studies for variants known in haematological disorders identified a variant in *EIF6* which is likely to be associated with a favorable bone marrow condition, a variant in *ASXL1* which warrants haematological surveillance, already scheduled in any SDS patient, and a variant in *GNAS*, which may explain the endocrinological abnormalities found, which are not part of the description of the syndrome. These types of data, especially if it is supported by functional studies, clearly suggest that the use of extended genome analysis can provide useful data for better patient management.

This was demonstrated here for SDS, but it may be extended to other disorders. Certainly, a consistent portion of phenotypic variability in patients sharing the same monogenic disease is caused by additional genomic alterations.

All of findings resulting from the second sub-study demonstrate that whole exome sequencing is a useful technique for identifying putative pathogenic genetic variants that could play as cofactors in addition to the pre-existence of the *SBDS* mutations, particularly in SDS patients who presented with leukemia, neutropenia, bone marrow hypocellularity and thrombocytopenia. By enabling early intervention with curative therapies, including allogeneic transplantation, a better ability to identify people with a high risk of developing leukemia and other hematological diseases in SDS has the potential to enhance clinical outcomes. Moreover, these findings provide a rational framework for clinical surveillance strategies.

In conclusion, the findings of this thesis underline the need of performing a comprehensive genomic analysis on SDS patients who present unexpected or unusual features. Whole exome sequencing and bioinformatics tools based on artificial intelligence are now readily available, raising the possibility that these methods may contribute to the well-known clinical variability reported with Mendelian diseases. These new data are likely, in the future, to also be relevant for personalized medicine and therapy in selected cases or groups of patients.

CHAPTER 7- REFERENCES

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SUPPLEMENTARY TABLES

Table 1 S. *Variants with good quality (QUAL≥30) and related to each HPO terms described in table (1) for UPN42*

HP:0001875 Neutropenia			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
SBDS	Pathogenic	c.258+2T>C	8.0
GFI1	VUS	c.925-12_925-5delCTCTCTCT	1.5
LRBA	VUS	c.7800C>T	1.5
MDM4	VUS	c.*3931T>G	1.5
AP3D1	VUS	c.*292A>T	1.5
HP:0005528 Bone marrow hypocellularity			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
SBDS	Pathogenic	c.258+2T>C	8.0
LBR	VUS	c.*117dupT	2.0
MDM4	VUS	c.*3931T>G	1.5
HP:0001738 Exocrine pancreatic insufficiency			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
SBDS	Pathogenic	c.258+2T>C	8.0
LIPA	Likely pathogenic	c.894G>A	6.0
ABCB4	VUS	c.3481C>T	2.5
DNMT 3B	VUS	c.*577C>A	1.5
HP:0001873 Thrombocytopenia			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
SBDS	Pathogenic	c.258+2T>C	8.0
RAG1	VUS	c.1346G>A	3.5
NFKB1	Likely benign	c.670A>G	3.0
LRBA	VUS	c.7800C>T	1.5
HP:0040088 Abnormal lymphocyte count			
Gene	**Classification**	HGVS_Coding	**eVai Pathogenicity_Score**
SBDS	Pathogenic	c.258+2T>C	8.0
LRBA	VUS	c.7800C>T	1.5
HP:0012189 Hodgkin lymphoma			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
CD27	VUS	c.*71_*72delTG	1.5
POLE	VUS	c.6747+39C>G	1.5
TCF4	VUS	c.73-831_73-802delGCTGCTGCTGCTGCTGCTGCTGCTGCT	2

Table 2 S. *Variants with good quality (QUAL≥30) and related to each HPO terms described in table (1) for UPN43*

HP:0001875 Neutropenia			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
SBDS	Pathogenic	c.258+2T>C	8.0
MMACHC	VUS	c.*860_*862delTCA	2.0
SLC35A1	VUS	c.294T>C	1.5
LRBA	VUS	c.4719A>G	1.5
HP:0005528 Bone marrow hypocellularity			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
SBDS	Pathogenic	c.258+2T>C	8.8
HP:0001738 Exocrine pancreatic insufficiency			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
SBDS	Pathogenic	c.258+2T>C	8.8
ABCB4	VUS	c.3481C>T	2.5
HP:0001873 Thrombocytopenia			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
SBDS	Pathogenic	c.258+2T>C	8.0
RAG1	VUS	c.1346G>A	3.5
NFKB1	Likely benign	c.670A>G	3.0
MSH3	VUS	c.162_188delTGCAGCGGCCGCAGCGGCCGCAGCGCC	3.0
MMACHC	VUS	c.*860_*862delTCA	2.0
SLC35A1	VUS	c.294T>C	1.5
LRBA	VUS	c.4719A>G	1.5
LARS2	VUS	c.1552G>A	1.5
EFCAB13	VUS	c.-405A>G	1.5
HP:0003025 Metaphyseal irregularity			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
SBDS	Pathogenic	c.258+2T>C	8.0
KMT2A	Likely pathogenic	c.10663G>A	5.5
ROR2	VUS	c.538A>G	4.0
PLCB3	VUS	c.29C>T	4.0
LTBP3	VUS	c.3702C>T	3.0
SPRTN	VUS	c.-40A>G	1.5
HP:0012758 Neurodevelopmental delay			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
SBDS	Pathogenic	c.258+2T>C	8.0
KMT2A	Likely pathogenic	c.10663G>A	5.5
ROR2	VUS	c.538A>G	4.0
SLC5A5	VUS	c.1096A>G	4.0
DHX37	VUS	c.2513A>G	3.5
SMPD1	VUS	c.872G>A	3.5
BTD	VUS	c.1270G>C	3.5

GNPAT	VUS	c.1985T>A	3.0
MSH3	VUS	c.162_188delTGCAGCGGCCGCAGCGGCCGCAGCGCC	3.0
BCORL1	VUS	c.*2C>T	3.0
GMPPB	VUS	c.*227T>A	3.0
CNTNAP2	VUS	c.416A>G	2.5
DHTKD1	VUS	c.240A>C	2.5
WASHC4	VUS	c.81A>C	2.5
SACS	VUS	c.470A>C	2.5
WDR62	VUS	c.3868A>G	2.5
BMPER	VUS	c.*1484delG	2.0
ASAH1	VUS	c.*176_*177delCT	2.0
MMACHC	VUS	c.*860_*862delTCA	2.0
RECQL4	Likely benign	c.1207T>C	2.0
SLC1A4	VUS	c.*1427_*1428insAAGGACTGCTCTGTGGAG	2.0
TBCK	VUS	c.*1513delG	2.0
B3GLCT	VUS	c.*1746_*1752delCTTCTAC	2.0
ELP4	VUS	c.*1492_*1493insTCTTTTAATCTGTGT	2.0
GPC4	VUS	c.*1314_*1315insC	2.0
CBL	VUS	c.*559_*560insCAA	2.0
CCDC85C	VUS	c.*1399_*1401delTTT	2.0
CCDC174	VUS	c.*851_*852insAGGG	2.0
DGCR2	VUS	c.-29_-28insGGGGTCACGCAGC	2.0
DGCR8	VUS	c.*1599_*1600insGGGGG	2.0
SMAD4	VUS	c.*5539_*5552delGCGCGCGCACACAC	2.0
SLC35A1	VUS	c.294T>C	1.5
PLOD3	VUS	c.*91T>A	1.5
DISC1	VUS	c.*4221T>G	1.5
SLC6A9	VUS	c.687C>T	1.5
WDR81	VUS	c.3039G>A	1.5
KDM6B	VUS	c.1197C>T	1.5
DEPDC5	VUS	c.2872C>T	1.5
		HP:0000028 Cryptorchidism	
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
KMT2A	Likely pathogenic	c.10663G>A	5.5
ALDH18A1	VUS	c.1115C>A	1
ARNT2	VUS	c.-89G>T	1
ATN1	Likely benign	c.1500_1508delGCAGCAGCA	1
ATN1	Likely benign	c.1506_1508dupGCA	0,5
CCDC141	VUS	c.422C>T	0,5
CDC73	VUS	c.*1144dupT	0
DHX37	VUS	c.2513A>G	3,5
DUSP6	VUS	c.260C>T	1,5
DUSP6	VUS	c.-187G>A	1

DYNC2H1	Likely benign	c.911A>T	0
EHMT1	Likely benign	c.1566T>G	0
EP300	VUS	c.5259C>T	0,5
FANCA	Likely benign	c.2151G>T	0
FREM2	Likely benign	c.4031G>A	0
IL17RD	VUS	c.499C>T	2,5
IL17RD	Likely benign	c.878C>T	0
LSS	Benign	c.1840C>T	0
RECQL4	Likely benign	c.1207T>C	2
RFWD3	Likely benign	c.1082C>T	0
ROR2	VUS	c.538A>G	4
RPGRIP1	Likely benign	c.10C>A	2
ZNF687	VUS	c.-166T>A	0
HP:0002719 Recurrent infection			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
SBDS	Pathogenic	c.258+2T>C	8.0
KMT2A	Likely pathogenic	c.10663G>A	5,5
IL21R	VUS	c.1124C>T	3
ACP5	Likely benign	c.299G>A	1
ADA2	Likely benign	c.1045G>A	1
ALDH18A1	VUS	c.1115C>A	0,5
ALOX12B	Likely benign	c.379C>T	1
ALOXE3	VUS	c.680C>T	1,5
ALOXE3	Likely benign	c.298C>G	1
ANTXR2	Likely benign	c.1360G>A	0
BTD	VUS	c.1270G>C	3,5
CYBC1	VUS	c.145C>T	0,5
DNAH1	Likely benign	c.4531G>A	0
IL7R	VUS	c.376A>C	0,5
LRBA	VUS	c.4719A>G	1,5
PKHD1	Likely benign	c.1736C>T	0
PLCG2	VUS	c.3646C>A	1
PRKDC	VUS	c.9938G>A	2,5
ROR2	VUS	c.538A>G	4
XIAP	Likely benign	c.606T>C	1
HP:0000431 Wide nasal bridge			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
KMT2A	Likely pathogenic	c.10663G>A	5,5
ADA2	Likely benign	c.1045G>A	1
EP300	VUS	c.5259C>T	0,5
FREM2	Likely benign	c.4031G>A	0
GNPAT	VUS	c.1985T>A	3
KDM6B	VUS	c.1197C>T	1,5
LTBP3	VUS	c.3702C>T	3

SOX18	VUS	c.801G>A	0,5
HP:0000316 Hypertelorism			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
KMT2A	Likely pathogenic	c.10663G>A	5,5
ADA2	Likely benign	c.1045G>A	1
CBX2	Likely benign	c.1021C>G	1
EHMT1	Likely benign	c.1566T>G	0
EP300	VUS	c.5259C>T	0,5
FANCA	Likely benign	c.2151G>T	0
LRP2	Likely benign	c.13250G>A	0
LRP2	Likely benign	c.3452C>T	0
HP:0000085 Horseshoe kidney			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
KMT2A	Likely pathogenic	c.10663G>A	5,5
ADA2	Likely benign	c.1045G>A	1
FANCA	Likely benign	c.2151G>T	0
SLX4	Likely benign	c.1372A>G	0
HP:0002474 Expressive language delay			
Gene	**Classification**	**HGVS_Coding**	**eVai Pathogenicity_Score**
EHMT1	Likely benign	c.1566T>G	0
SDHA	VUS	c.*175dupG	2
WARS1	VUS	c.1230C>T	0

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CASE REPORT
published: 12 August 2022
doi: 10.3389/fgene.2022.896749

Case Report: Heterozygous Germline Variant in *EIF6* Additional to Biallelic *SBDS* Pathogenic Variants in a Patient With Ribosomopathy Shwachman–Diamond Syndrome

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Background: Shwachman–Diamond syndrome (SDS) is a rare autosomal recessive ribosomopathy mainly characterized by exocrine pancreatic insufficiency, skeletal alterations, neutropenia, and a relevant risk of hematological transformation. At least 90% of SDS patients have pathogenic variants in *SBDS*, the first gene associated with the disease with very low allelic heterogeneity; three variants, derived from events of genetic conversion between *SBDS* and its pseudogene, *SBDSP1*, provided the alleles observed in about 62% of SDS patients.

Methods: We performed a reanalysis of the available WES files of a group of SDS patients with biallelic *SBDS* pathogenic variants, studying the results by next bioinformatic and protein structural analysis. Parallelly, careful clinical attention was given to the patient focused in this study.

Results: We found and confirmed in one SDS patient a germline heterozygous missense variant (c.100T>C; p.Phe34Leu) in the EIF6 gene. This variant, inherited from his mother, has a very low frequency, and it is predicted as pathogenic, according to several *in silico* prediction tools. The protein structural analysis also envisages the variant could reduce the binding to the nascent 60S ribosomal.

Conclusion: This study focused on the hypothesis that the *EIF6* germline variant mimics the effect of somatic deletions of chromosome 20, always including the locus of this gene, and similarly may rescue the ribosomal stress and ribosomal dysfunction due to *SBDS* mutations. It is likely that this rescue may contribute to the stable and not severe hematological status of the proband, but a definite answer on the role of this *EIF6* variant can be obtained only by adding a functional layer of evidence. In the future, these results are likely to be useful for selected cases in personalized medicine and therapy.

Keywords: Shwachman–Diamond syndrome, *EIF6*, *SBDS*, whole-exome sequencing, case report

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Genetics of Common and Rare Diseases, a section of the journal Frontiers in Genetics

Received: *09 May 2022*
Accepted: *23 June 2022*
Published: *12 August 2022*

Citation:
Taha I, Foroni S, Valli R, Frattini A, Roccia P, Porta G, Zecca M, Bergami E, Cipolli M, Pasquali F, Danesino C, Scotti C and Minelli A (2022) Case Report: Heterozygous Germline Variant in EIF6 Additional to Biallelic SBDS Pathogenic Variants in a Patient With Ribosomopathy Shwachman–Diamond Syndrome. Front. Genet. 13:896749. doi: 10.3389/fgene.2022.896749

INTRODUCTION

Shwachman–Diamond syndrome (SDS) is a rare, autosomal recessive disease (OMIM #260400) included among inherited bone marrow failure syndrome (IBMFS). It is primarily characterized by neutropenia, exocrine pancreatic insufficiency, skeletal alterations, and an increased risk to develop hematological malignancies, including myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (Dror et al., 2011; Nelson and Myers, 2018). The disease-causing gene is *SBDS*, localized at 7q11.21 (Boocock et al., 2003), and a large majority of patients carry biallelic pathogenic variants in *SBDS*, the detection of which confirms the clinical diagnosis. Typically, mutation analysis of exon 2 identifies, in at least 90% of SDS patients, one of three common mutations, (c.183_184delTAinsCT, c.258+2T>C, and c.258+2T>C in addition to c.183_184delTAinsCT in the same allele). Two of these common mutations are observed concomitantly in approximately 62% of SDS patients (Nelson and Myers, 2018).

The approach of whole-exome sequencing (WES) has disclosed genetic heterogeneity for SDS. Indeed, biallelic mutations in DNAJ homolog subfamily C member 21 (*DNAJC21*) (Tummala et al., 2016; Dhanraj et al., 2017), in GTPase Elongation Factor-like (*EFL1*) (Stepensky et al., 2017; Tan et al., 2018; Tan et al., 2019; Lee et al., 2021), or heterozygous mutations in the 54-kDa signal recognition particle (*SRP54*) (Carapito et al., 2017) have been reported in association with SDS or SDS-like phenotype in some patients.

When *SRP54* is involved in the co-translation protein-targeting pathway, the other genes code for proteins involved in the ribosome biogenesis pathway as SBDS: 1) DNAJC21 is involved in the pre-rRNA processing, and it is implicated in the late 60S maturation in the cytoplasm; 2) EFL1 interacts with SBDS to evict the anti-association factor eIF6 from the ribosomal subunit 60S in the final step of ribosome maturation (Weis et al., 2015). The removal of eIF6 from the 60S subunit allows the 80S ribosome assembly (Ceci et al., 2003).

In SDS, because of the loss of SBDS activity, eIF6 remains bound to the 60S subunit and prevents its joining to the 40S subunit, resulting in decreased ribosomal subunit joining and reduced translation efficiency. The impairment of ribosome biogenesis links SDS to the group of bone marrow failure disorders, called ribosomopathies, associated with mutations in genes related to ribosomal biogenesis and function. Ribosomopathies are characterized by ribosome dysfunction producing marrow failure and cancer predisposition (De Keersmaecker et al., 2015).

Focusing on the SDS predisposition to hematological malignancies, a growing interest in eIF6 and its role in ribosome maturation prompted two different types of studies. The first concerns the interstitial deletion of the long arm of chromosome 20, del(20)(q), identified by periodic bone marrow monitoring of SDS patients; this clonal abnormality always includes the loss of the *EIF6* locus (Pressato et al., 2015). Clinical follow-up of SDS patients showed that del(20)(q), without additional clonal chromosomal abnormalities, correlates with a lower risk of developing MDS and/or AML, suggesting that the loss of one copy of *EIF6* gene restores ribosome homeostasis in the context of reduced SBDS activity (Valli et al., 2013; Khan et al., 2020; Khan et al., 2021). The del(20)(q) has also been associated with an increased number of burst-forming unit-erythroid (BFU-E) colonies in cases of paternal deletion (Nacci et al., 2017).

The second study monitored the mutational status on multiple bone marrow samples of patients with SDS. By using NGS sequencing, recent research demonstrated the frequent development of hematopoietic clones carrying a heterozygous mutation in *EIF6* and *TP53* genes. Functional studies observed the inactivation of eIF6 and the corresponding improvement of the less effective SDS translation, supporting the hypothesis of improved clone fitness (Kennedy et al., 2021; Tan et al., 2021).

All these studies unveiled that SDS represents another genetic disease in which a somatic rescue mechanism occurs, either through a del(20)(q), always encompassing the *EIF6* locus, or through *EIF6* heterozygous variants.

As a part of a larger study of whole-exome sequencing (WES) on SDS patients carrying biallelic causative *SBDS* pathogenic variants, aimed to understand if other germline variants could explain the large clinical variability among the patients (Morini et al., 2019), we identified a single case carrying a heterozygous germline variant (c.100T>C, p.Phe34Leu) in *EIF*. We postulate, with strong support from structural data, that this variant impairs protein activity, thus reducing the ribosomal dysfunction, similarly to deletions of chromosome 20, and, likely, resulting in a milder and stable hematological picture in the reported patient.

MATERIALS AND METHODS

Case Report

Our patient, a 23-year-old male (born in 1998), identified as UPN 2 (unique patient number) in the previous work by our group (Valli et al., 2019), is the second child of healthy non-consanguineous Italian parents. UPN 2 was diagnosed as affected by SDS in 2001 based on the presence of neutropenia and pancreatic insufficiency.

Since diagnosis, an annual follow-up has been performed at the pediatric onco-hematology unit. Over the last 10 years, the patient has maintained a stable hematological picture.

At the latest clinical evaluation (2021), his height was 155.0 cm, his weight 50.0 kg, and his chest circumference 77.5 cm, all at 25–30 centiles of height and weight distribution of SDS male/female patients (personal communication by M. Cipolli and G. Tridello). At the same time, no pathological changes were observed in both lower limbs, and normal bone density was reported. Similarly, no structural changes in the thoracic cage were present. His school performance was low average. At the same clinical evaluation of 2021, hemoglobin and RBC counts were normal (14.9, normal value, nv: 13.2–17.3 g/dl and 4.35×10^6 per µl, nv $4.30–5.70 \times 10^6$ per µl, respectively). The neutrophil count was low (0.9×10^3, nv: $2.0–8.0 \times 10^3$ per µl, and ranging between 0.5 and 0.7 $\times$ 10^3 per µl in previous tests), lymphocyte count was normal (1.8×10^3µL, nv: $1.5–4.0 \times 10^3$ per

µl), and platelets were slightly reduced (120 × 10³ per µl, nv: 150–400 × 10³ per µl, ranging between 100 and 130 × 10³ per µl in previous tests). Bone marrow biopsy confirmed, as the previous controls, a stable cellularity (20%), and immunostaining with anti-p53 antibody was consistently negative. No evidence of any change suggesting myelodysplasia was reported.

Routine biochemical tests were within normal limits; serum amylase and pancreatic isoamylase were low, 14.0 mU/ml and 5.0 mU/ml, respectively (nv: 25.0–125.0 mU/ml and 8.0–53.0 mU/ml, respectively). Serum lipase was within normal limits: 36.0 mU/ml (nv: 8.0–58.0 mU/ml). Creon tablets as a treatment for the exocrine pancreatic insufficiency were prescribed. S-Parathyroid hormone (PTH) levels were slightly elevated during the last two checks, corresponding to 112.3 and 84.5 pg/ml values (nv: 12,0–72,0 pg/ml). Vitamin D, calcium, and potassium were at the normal levels. The patient had received his third dose of COVID-19 vaccine without any major complication.

Similarly, a recent survey carried out by an American SDS Registry, confirmed no worrying clinical effect in three SDS patients who have been vaccinated against COVID-19 (Galletta et al., 2022).

Cytogenetics

Repeated conventional and molecular cytogenetic analyses were performed on bone marrow yearly since 2002, almost regularly, up to 2021. The methods used included routine cytogenetics, Fluorescent *In Situ* Hybridization (FISH) on metaphases and nuclei with informative probes and libraries, and array-based Comparative Genomic Hybridization (a-CGH), according to the methods reported in Pressato et al. (2015).

Molecular and Bioinformatics Investigations

In 2015, we performed WES on 16 DNA samples obtained from blood samples of SDS patients after their acceptance to the project. Samples eligible for WES held as key features a concentration of 50 ng/ul, a ratio 260/280λ and 260/230λ, respectively, at least equal to 1.8 and 1.8–2.1, and a good integrity evaluated by agarose gel electrophoresis. Sequencing libraries of the samples were prepared using the exome enrichment kit *Agilent SureSelectXT Human All Exon V5+UTRs*. WES was performed on the HiSeq 1,000 (Illumina, 2 bp × 100 bp). The processing of sequencing data was based on the ISAAC pipeline (Raczy et al., 2013); for data alignment and for variants calling, we used, respectively, the ISAAC aligner and ISAAC variant caller. From the samples alignment we obtained a mean coverage of 65% and a coverage >20X corresponding to 91% of the complete exome sequences. For the annotation and the analysis of the variants in the variant call format (VCF) files, generated by WES for each sample, the expert variant interpreter (eVai) platform (https://www.engenome.com/evai/) (Nicora et al., 2022) was used.

We confirmed the relevant variants by Sanger sequencing. The primers used for the amplification and sequencing were designed using Primer3web (https://primer3.ut.ee/). PCRs were performed in 25 µL of reaction volume containing 50 ng of genomic DNA, 0.5 µm primers, 2 µl dNTP Mixture (2.5 mm each), 2.5 µl 5X PrimeSTAR GXL Buffer, and 1.25U PrimeSTAR GXL DNA Polymerase (TAKARA BIO INC.). PCR products were checked by 2% agarose gel electrophoresis and underwent clean-up prior to sequencing using the Applied 3500 DX Genetic Analyzer sequencer with BrilliantDye Terminator Cycle sequencing kit (NimaGen). The sequencing data were analyzed by Finch TV software.

Structural Analysis

For structural visualization and evaluation of the effect of the Phe34Leu mutation, the model of eIF6 was extracted from PDB code 6LSS (pre-60 ribosome). This model had originally been generated by SWISS-MODEL (Waterhouse et al., 2018) and docked into the electron density of human pre-60S ribosomal particles by rigid-body fitting, followed by extensive manual rebuilding in COOT with a final local resolution comprised between 3.1 and 4.8 Å (Liang et al., 2020).

RESULTS

Cytogenetic Findings

Bone marrow karyotype analysis was normal up to 2007; then, from 2008 to 2010 a small clone was detected carrying an isochromosome for the long arm of chromosome 7, i(7)(q10), which is one of the most frequent clonal chromosome anomalies in the bone marrow of SDS patients: the abnormal cells ranged from 1.7 to 7.6%, according to different methods (conventional cytogenetics or FISH with different probes) or different times of analysis. On DNA from a sample with 2.9% of cells with the i(7)(q10), a-CGH was performed; it did not reveal the chromosome 7 abnormality due to the small size of the clone, nor any other abnormality. The i(7)(q10) clone then disappeared, and was not found in any subsequent analysis from 2011 to 2020; no structural abnormality of chromosome 20 was ever identified.

Molecular and Bioinformatics Analysis

The clinical diagnosis of SDS was confirmed by the presence of one of the common mutations [c.258+2T>C, p.Cys84fs3, paternal] and a second variant [c.356G>A, p.Cys119Tyr, maternal] in *SBDS*. The latter was included by Finch et al. (2011) among disease-associated missense variants since, on the basis of functional studies, it was evaluated as a mutation affecting SBDS protein stability.

When reviewing our WES file of UPN2 using the eVai platform, we first filtered the results searching for variants in other genes related to SDS or SDS-like phenotypes, *DNAJC21*, *EFL1*, and *SRP54*, no relevant variants were found. Then, checking for variants in *EIF6* gene, we found a heterozygous single nucleotide substitution causing a missense change (c.100T>C; p.Phe34Leu). Sanger sequencing confirmed the variant for the patient and assessed its maternal origin (**Figure 1A**). According to the Genome Aggregation Database (gnomAD), the variant is a rare one as its frequency is 0.0002002.

These results were obtained in DNA from a blood sample, but we checked the variant in DNAs also from eight bone marrow

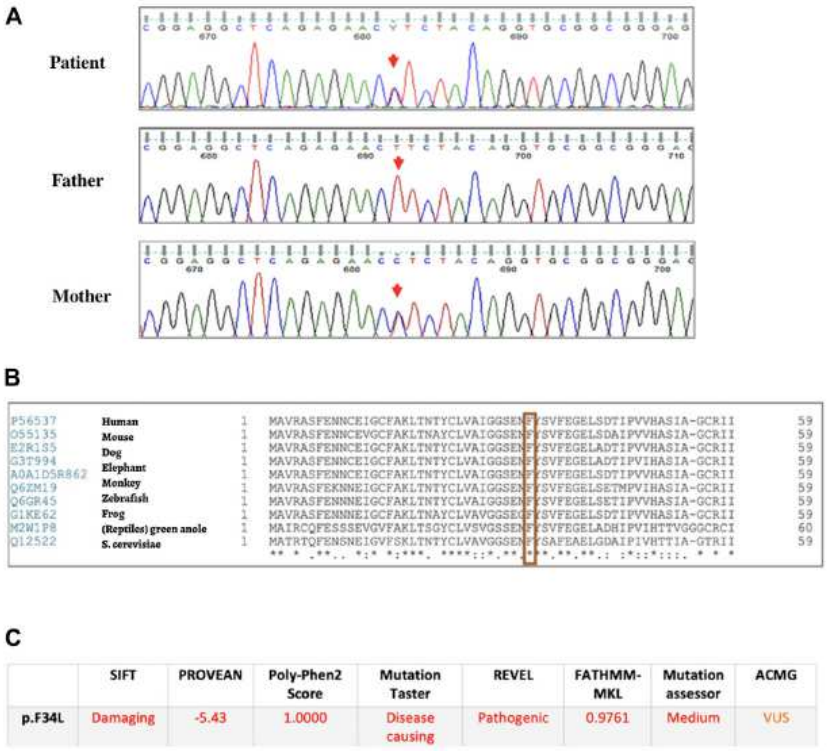

FIGURE 1 | Sequencing confirmation of the *EIF6* variant. **(A)** Part of electropherogram from the proband and their parents, showing the substitution T>C in the heterozygous status at the position c.100 maternal inherited; **(B)** protein sequence alignment using UniProt shows absolute conservation of F34 throughout different species (human, mouse, dog, elephant, monkey, zebrafish, frog, reptiles, and *S. cerevisiae*); **(C)** *in silico* analysis of the p.Phe43Leu variant using different prediction tools.

specimens obtained at regular follow-ups (the latest one in 2021): all of these showed the stable presence of the heterozygous variant, with a ratio 1:1 to normal allele.

Protein sequence alignment using UniProt (www.uniprot.org) showed that the amino acid Phenylalanine in position 34 is highly conserved among different species such as human, mouse, dog, elephant, monkey, zebrafish, frog, reptiles, and *S. cerevisiae* (**Figure 1B**). The variant functional predictions are entered in **Figure 1C**.

We then filtered the WES results for the presence of variants in genes related to the development of MDS or leukemia, as reported by several authors (Lindsley, 2017; Steensma, 2017; Armstrong et al., 2018; Bluteau et al., 2018; Obrochta and Godley, 2018; Kennedy et al., 2021). The variants with a frequency ≤0.01 were validated by Sanger sequencing in the patient and his parents. These variants are entered in **Table1** with their genome position, amino acid change, zygosity, allele frequency, and pathogenicity prediction, according to various specific tools and parental origin.

Structural Analysis

The protein eIF6 consists of five *quasi*-identical alpha/beta subdomains arrayed about a five-fold axis of pseudosymmetry (**Figure 2A**). Residue F34 (blue sticks) is the core part of a chain of hydrophobic interactions mediated by a cluster of five aromatic residues (F7, F16, F34, Y35, and F38) (pink sticks) belonging to the N-terminal domain and one, F205, belonging to the C-terminal domain (**Figure 2B**). This residue arrangement, involving helix 2A and β-strand 2E, where the group of four residues, F34, Y35, F38, and F205, are located, respectively (nomenclature according to (Groft et al., 2000), contributes to stabilize the "velcro" strategy adopted in the pentein fold of eIF6 (Fülöp and Jones, 1999), where β-strand 2E at the C-terminus hydrogen bonds with a β-strand from the N-terminal subdomain. The flat protein surfaces, thus, formed are necessary for interaction with ribosomal protein L23, a component of the 60S subunit, on one side, and with GTPBP4 (nucleolar GTP-binding protein 1), also involved in the 60S biogenesis, on the other (**Figures 2C,D**). The F34L variant lacks the central phenylalanine of the cluster, interrupts this regular pattern and introduces clashes, represented by solid red disks in **Figures 2E,F**, where both leucine rotamers are considered. Despite the similar hydrophobic nature of the two residues, the reduction of the side chain size and the interruption of the aromatic stack explain why analysis by I-mutant predicts a Gibbs free energy difference (ΔΔG) between the mutant and the wild-type of −0.87 kcal/mol. This result is evidence of a large decrease

TABLE 1 | Variants found in UPN 2 with a frequency ≤0.01 in genes related to MDS and AML.

Chr: Position	Gene	cDNA (a.a)	Effect	Zyg	MAF	*In silico* prediction tools						Parental Origin
						SIFT	Mutation assessor	FATHMM	MutationTaster	M-CAP	REVEL	ACMG	
20: 31,024,146	ASXL1	c.3631G>A (p.Asp1211Asn)	Missense	Het	0.0	Damaging	Medium	Tolerated	Polymorphism	Tolerated	Benign	VUS	Father
9:5,022,031	JAK2	c.44C>T (p.Ser15Phe)	Missense	Het	0.0000159	Damaging	Low	Tolerated	Polymorphism	Possibly pathogenic	Benign	VUS	Father
20: 57,474,003	GNAS	c.2149G > T (p.Glu717Ter)	LoF	Het	0.0	NA	NA	Damaging	Disease causing	Possibly pathogenic	NA	Pathogenic	Father

MDS, myelodysplastic syndromes; AML, acute myeloid leukemia; Chr, chromosomal; A.A, amino acid change; LoF, loss of function; Zyg, zygosity; Het, heterozygous; MAF, minor allele frequency; VUS, variant of uncertain significance; SIFT, sorting intolerant from tolerant (https://sift.bii.a-star.edu.sg); FATHMM, functional analysis through hidden Markov models (http://fathmm.biocompute.org.uk); M-CAP, Mendelian clinically applicable pathogenicity (http://bejerano.stanford.edu/mcap); REVEL, rare exome variant ensemble learner (https://sites.google.com/site/revelgenomics); ACMG, American College of Medical Genetics

in protein stability. We can, therefore, envisage that the F34L variant could impair eIF6 folding, with a reduction in binding to the nascent 60S ribosomal subunit through protein L23 and a possible consequent reduction in binding of protein GTPBP4, which is also involved in ribosomal biogenesis.

DISCUSSION

Three main features characterize SDS: bone marrow failure, pancreatic exocrine dysfunction, and skeletal abnormalities (Dror et al., 2011). In addition, a significant percentage of SDS patients may develop serious hematological complications, requiring, in some cases, hematopoietic stem cell transplantation, a procedure associated with a requirement for specific protocols for this disease (Donadieu et al., 2012). A challenge for the surveillance of the disease is the early identification of risk factors relevant to the evolution of the hematological picture (Furutani et al., 2022). An extensive study on a French cohort of 102 patients postulated that the combination of a young age at diagnosis with low values of specific hematologic parameters, at the time of diagnosis and later constitutes a negative prognostic factor. This combination was a risk factor stronger than any of the other nine single variables taken into consideration alone, as transient cytopenia, low value of neutrophils, hemoglobin, platelets, or *SBDS* genotype. The latter was confirmed not to be linked to significant differences in hematologic parameters and other nonhematologic features, both in patients harboring two common mutations or in patients with one common and one rare mutation (Donadieu et al., 2012).

Although the risk for developing MDS and AML is not related to the type of *SBDS* mutations, it can be favorably modified by other genetic factors as the presence of specific chromosomal abnormalities, in particular, the i(7)(q10) and del(20)(q) if they are the only abnormality present (Minelli et al., 2009; Khan et al., 2021).

When del(20)(q) is considered, cytogenetic studies, made over a long period of time on the SDS Italian cohort, confirmed the benign prognostic significance of this abnormality. Indeed, the patients carrying del(20)(q) consistently showed a mild hypoplastic bone marrow picture, no or mild neutropenia, anemia, and thrombocytopenia (Pressato et al., 2012; Valli et al., 2019).

In all cases, del(20)(q) includes the loss of the *EIF6* locus and, consequently, in the bone marrow clones with the chromosomal abnormality, only one allele expressing *EIF6* is present in the bone marrow clones with the chromosomal abnormality. This condition has been studied *in vivo* on mice demonstrating that knockout heterozygous animals presented reduced eIF6 levels as expected, while preserving sufficient nucleolar eIF6 and regular ribosome biogenesis. In the liver cells, a larger amount of 80S in polysomal profiles suggested a lower efficiency in initiation of translation (Gandin et al., 2008).

Common events in SDS patients are the development of many somatic clones in the bone marrow with variants in either *EIF6* or *TP53* genes, producing different effects: i) variants in *EIF6* have a

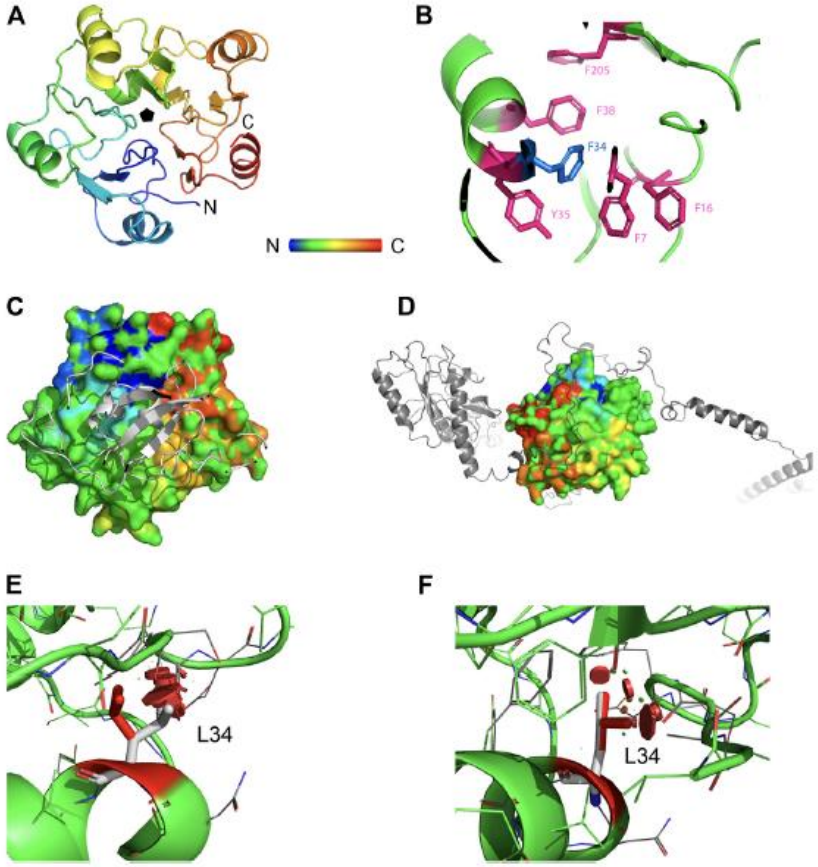

FIGURE 2 | Structural analysis of eIF6. **(A)** Rainbow-colored (blue: N-terminus, red: C-terminus) ribbon representation of eIF6, consisting of five quasi-identical alpha/beta subdomains arrayed about a five-fold axis of pseudosymmetry. **(B)** Residue F34 (blue sticks) is the core part of a chain of hydrophobic interactions mediated by a cluster of five aromatic residues (F7, F16, F34, F38, and Y35) belonging to the N-terminal domain and one, F205, belonging to the C-terminal domain (pink sticks). **(C)** Surface representation of eIF6 interacting with ribosomal protein L23 (gray ribbon). **(D)** Surface representation of eIF6 interacting with ribosomal protein GTPBP4 (gray ribbon). **(E,F)** Effect of the F34L mutation: red solid disks represent clashes between the two rotamers of L34 and neighboring residues.

compensatory role on the impact of SBDS deficiency, in turn reducing the risk of developing MDS and AML by improving the ribosome maturation, enhancing translation, and reducing p53 upregulation (Kennedy et al., 2021), ii) variants in *TP53* act in a different pathway and decrease tumor suppressor checkpoint activation. Clones acquiring only variants in both *TP53* alleles are associated with the development of hematological malignancies (Kennedy et al., 2021).

Furthermore, higher p53 protein levels, linked to inhibition of osteogenesis, have been found in SDS patient's osteoblasts, in correlation with the skeletal anomalies of patients (Frattini et al., 2021).

Relevantly, the *EIF6* clonal variants in SDS patients were not observed in the presence of leukemic evolution or serious bone marrow dysfunction, confirming a positive impact on the hematological alterations determined by the *SBDS* genotype (Kennedy et al., 2021).

The results on humans fit the yeast model proposed by Menne et al. (2007) who demonstrated that missense variants in *Tif6* can bypass the fitness defect resulting from a lack of *Sdo1* through the reduction of *Tif6* binding to the 60S ribosome subunit.

Here, we report a patient, UPN 2, in whom we found a heterozygous germline variant, c.T>C (p.Phe34Leu), in *EIF6*. This variant is rare according to gnomAD and the amino acid Phe34 is extensively conserved (**Figure 1B**). To the best of our knowledge, this is the first patient in whom a constitutional pathogenic *EIF6* variant is associated with biallelic *SBDS* mutations. In UPN 2, the missense variant in *EIF6* is a germline variant thus, at least in theory, its effect in ameliorating the consequences of *SBDS* mutations is expected to have started to act from zygote and to be widespread to all tissues.

Available clinical data suggest that stature (the patient is at low centiles for SDS patients), pancreatic function (the patient is still on therapy with pancreatic enzymes), and cognitive development are not strongly and favorably influenced. As far as the skeletal features are concerned, osteoblasts from UPN 2 did not differ with respect to osteoblasts from a group of 13 SDS patients studied by Frattini et al. (2021) who demonstrated lower mineralization capacity and lower expression of genes responsible for osteoblastogenesis.

Conversely, hematological data and bone marrow picture, over the last 10 years, have been stable; hemoglobin, platelets, and lymphocytes have consistently been within normal limits and the patient never needed any kind of therapy. The patient has moderate cytopenia, which does not appear to have ever required transfusions or the use of hematopoietic growth factors.

Only mild bone marrow alterations were present, and no evidence of any worsening has been reported so far, during the course of the disease. In addition, negative anti-p53 antibody reactivity has been repeatedly observed.

Similarly, Tan et al. (2021) reported an *SBDS*-mutated patient with a somatic bone marrow variant in *EIF6* (c.182G>T, p.Arg61Leu), showing stable and mild hematological features. When studying this patient, they interpreted the variant as an example of a somatic genetic rescue. The Arg61Leu variant in fact rescued the defect of Sdo1-deficient yeast cells and the larval lethality of Sbds-deficient *Drosophila* (Tan et al., 2021). We expected that the variant p.Phe34Leu, identified in our patient, could have a similar effect as Arg61Leu, predicting a reduced binding to the nascent 60S ribosome, but it needs to be proven.

Our findings, on a germinal level, might agree with the hypothesis that somatic *EIF6* mutations, observed by Kennedy and others, produce a compensatory effect for the pre-existing germline *SBDS* mutations, thus reducing the risk of developing malignant neoplasia. Among these somatic mutations, missense ones were predominantly located in regions encoding conserved secondary structure motives, exactly like the p.Phe34Leu variant described here, which is a newly described one (Kennedy et al., 2021).

In all SDS patients, the diagnosis prompts compulsory monitoring, over time, of the most relevant symptoms, including exocrine pancreatic dysfunction, especially bone marrow failure (Valli et al., 2017). In our case, this program of surveillance will produce a large amount of useful information not only to manage the clinical problems of the patient but also to compare him with the group of other SDS patients, whose exome analysis did not reveal any significant *EIF6* variants.

If the periodic clinical check will confirm a stable and positive situation, particularly concerning the hematological picture, we can confirm that we have observed a human example of somatic genetic rescue through *EIF6* mutations comparable to yeast and *Drosophila* models.

Undoubtedly, our observations, supported by genetic considerations and *in silico* simulations, may be of actual use for clinical interpretation only when experimentally validated.

The study of the effect of p.Phe34Leu variant could also be improved by using the molecular dynamics simulation approach, as previously performed for EFL1 (Delre et al., 2020).

To check the presence, in addition to the *EIF6* variant, of any other variants in genes involved in MDS or AML (Lindsley, 2017; Steensma, 2017; Armstrong et al., 2018; Bluteau et al., 2018; Obrochta and Godley, 2018; Kennedy et al., 2021), we analyzed the exome file of UPN 2 and three genes, *ASXL1*, *JAK2*, and *GNAS*, were identified as bearing a mutation which might be relevant for the risk of MDS/AML (**Table1**).

ASXL1: Several studies showed that *ASXL1* somatic mutations are among the most frequent variants in all subtypes of myeloid malignancies including MDS (11–21% cases), AML (5–11%), myeloproliferative neoplasm (MPN), and chronic myelomonocytic leukemia (CMML) (40–50%) (Boultwood et al., 2010; Thol et al., 2011; Fujino and Kitamura, 2020). *ASXL1* mutations accelerate myeloid malignancies and are always associated with poor prognosis (Gelsi-Boyer et al., 2012).

Confirmatory evidence of *ASXL1* relevance in hematological malignancies was produced by Wang et al. (2014) who showed that heterozygous genetic *ASXL1*-knockout mice ($Asxl1^{+/-}$) developed MDS/MPN and by Abdel-Wahab et al. (2013), who reported that hematopoietic cell-specific deletions of Asxl1 induced an MDS-like disease. Germinal *de novo* nonsense or frameshift mutations in *ASXL1* cause the Bohring–Opitz syndrome (Bedoukian et al., 2018), and a few reports describe missense germline mutations, in association with familial recurrence of different hematological malignancies.

Hamadou and coworkers reported the mutation p.Arg402Gln in two Tunisian sisters affected by NHL (tonsil large B-cell lymphoma and gastric MALT lymphoma) (Hamadou et al., 2016); Seiter and coworkers reported a father and son, both carrying the p.Asn968Ser mutation, who developed MDS then evolving in AML at age 75 and 46, respectively (Seiter et al., 2018).

Zebisch and coworkers described a family in which a 60-year-old female affected with MDS was transplanted from her 58-year-old apparently healthy sister; the graft function was poor, and laboratory investigations demonstrated the presence of the same *ASXL1* mutation, p.Pro808His, in the donor sister (Zebisch et al., 2020).

An *ASXL1* mutation p.Gly704Arg is present in a family (a 2-year-old child and her 54-year-old paternal uncle, both affected with primary myelofibrosis) included in the study of Andres-Zayas and coworkers on germline predisposition for leukemia (Andrés-Zayas et al., 2021).

In our patient, the missense variant p.Asp1211Asn is interpreted as pathogenic only by SIFT (https://sift.bii.a-star.edu.sg) (Vaser et al., 2016) (**Table1**), and the previously reported germinal *ASXL1* mutations were reported as damaging or disease causing by SIFT and MutationTaster, respectively (https://www.mutationtaster.org) (Schwarz et al., 2014), while other tools classified them as benign.

Based on available evidence, missense germinal mutations in *ASXL1* might act as a risk factor for developing hematological malignancies; topics such as penetrance or anticipation can be discussed only when a much larger number of cases have been studied (Seiter et al., 2018). At present hematological surveillance in carriers of germinal missense mutations in *ASXL1* seems a reasonable management of the problem, even if the functional significance of the mutation is not fully defined.

JAK2: The *JAK2* variant, p.Ser15Phe, is interpreted as pathogenic only in one out of the seven prediction tools used (**Table 1**); it falls at the N-terminus outside the most functionally relevant domains reported by Benton and coworkers (Benton et al., 2019). Reports about germinal *JAK2* mutations are rare (Goldin et al., 2009; Park et al., 2020) and quoted mutations are described to be pathogenic when present in bone marrow as somatic mutations (Klampfl et al., 2013; Tefferi, 2016).

GNAS: The *GNAS* variant p.Glu717Ter, found in our patient and paternally inherited, is interpreted as pathogenic by all four

applicable prediction tools (**Table1**). Also, for *GNAS* reported evidence underlines its involvement in both genetic and hematological diseases.

Activating gain-of-function mutations are known to cause Mendelian disorders as polyostotic fibrous dysplasia and McCune–Albright syndrome (Robinson et al., 2016). *GNAS* somatic mutations have been recurrently found in hematological disorders such as MDS and lymphoma (Bejar et al., 2011; Xie et al., 2014). In addition, *GNAS* germline mutations are related to different types of pseudohypoparathyroidism (PHP-Ia,-Ib,-Ic) and pseudopseudohypoparathyroidism (PPHP). PHP and PPHP are autosomal dominant conditions and are associated with a lack of expression of Gsα if the pathogenic variant inactivates maternal or paternal alleles, respectively (Haldeman-Englert et al., 1993). As the clinical data on our patient include increased PTH, a careful evaluation for endocrinological condition was suggested to the family, and it is still in progress.

CONCLUSION

UPN 2 was diagnosed with SDS by targeted mutation analysis. WES studies for variants known in hematological disorders identified a variant in *EIF6* which is likely to be associated with a favorable bone marrow condition, a variant in *ASXL1* which warrants hematological surveillance, already scheduled in any SDS patient, and a variant in *GNAS*, which may explain the endocrinological abnormalities found, which are not part of the description of the syndrome.

These types of data, especially if it is supported by functional studies, clearly suggest that the use of extended genome analysis can provide useful data for better patient management.

This was demonstrated here for SDS, but it may be extended to other disorders. Certainly, a consistent portion of phenotypic variability in patients sharing the same monogenic disease is caused by additional genomic alterations. The progress in identifying these changes will help walk on the path of personalized medicine.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Molecular investigation and Sanger sequencing: IT, SF, AF, and AM; bioinformatic work and writing the manuscript: IT, AM, CS, and CD; clinical data: MZ, EB, and MC; modelling of proteins: CS; cytogenetic data: RV, PR, GP, and FP.

FUNDING

We gratefully acknowledge the financial support by the AISS (Associazione Italiana Sindrome di Shwachman) to AM. This research was supported by a Grant from the Italian Ministry of Education, University and Research (MIUR) to the Department of Molecular Medicine of the University of Pavia under the initiative "Dipartimenti di Eccellenza (2018–2022)". This work was supported by the grants from Ricerca Corrente to Fondazione IRCCS Policlinico San Matteo (RCR 08045818).

ACKNOWLEDGMENTS

The authors thank enGenome for improving the bioinformatics analysis. The authors specially thank the Italian Agency for Development and Cooperation (AICS), the Center of International Cooperation and Development (CICOPS) at University of Pavia, and Partnership for Knowledge (PfK) initiative for offering a Ph. D scholarship in Translational medicine to IT. This work is part of the Ph. D thesis of IT.

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Phenotypic Variation in Two Siblings Affected with Shwachman-Diamond Syndrome: The Use of Expert Variant Interpreter (eVai) Suggests Clinical Relevance of a Variant in the KMT2A Gene

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Citation: Taha, I.; De Paoli, F.; Foroni, S.; Zucca, S.; Limongelli, I.; Cipolli, M.; Danesino, C.; Ramenghi, U.; Minelli, A. Phenotypic Variation in Two Siblings Affected with Shwachman-Diamond Syndrome: The Use of Expert Variant Interpreter (eVai) Suggests Clinical Relevance of a Variant in the KMT2A Gene. *Genes* **2022**, *13*, 1314. https://doi.org/10.3390/genes13081314

Academic Editors: Manuela Priolo and Marco Tartaglia

Received: 17 June 2022
Accepted: 20 July 2022
Published: 23 July 2022

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Abstract: Introduction. Shwachman-Diamond Syndrome (SDS) is an autosomal-recessive disorder characterized by neutropenia, pancreatic exocrine insufficiency, skeletal dysplasia, and an increased risk for leukemic transformation. Biallelic mutations in the *SBDS* gene have been found in about 90% of patients. The clinical spectrum of SDS in patients is wide, and variability has been noticed between different patients, siblings, and even within the same patient over time. Herein, we present two SDS siblings (UPN42 and UPN43) carrying the same *SBDS* mutations and showing relevant differences in their phenotypic presentation. Study aim. We attempted to understand whether other germline variants, in addition to *SBDS*, could explain some of the clinical variability noticed between the siblings. Methods. Whole-exome sequencing (WES) was performed. Human Phenotype Ontology (HPO) terms were defined for each patient, and the WES data were analyzed using the eVai and DIVAs platforms. Results. In UPN43, we found and confirmed, using Sanger sequencing, a novel de novo variant (c.10663G > A, p.Gly3555Ser) in the *KMT2A* gene that is associated with autosomal-dominant Wiedemann–Steiner Syndrome. The variant is classified as pathogenic according to different in silico prediction tools. Interestingly, it was found to be related to some of the HPO terms that describe UPN43. Conclusions. We postulate that the *KMT2A* variant found in UPN43 has a concomitant and co-occurring clinical effect, in addition to *SBDS* mutation. This dual molecular effect, supported by in silico prediction, could help to understand some of the clinical variations found among the siblings. In the future, these new data are likely to be useful for personalized medicine and therapy for selected cases.

Keywords: Shwachman-Diamond Syndrome; *SBDS*; *KMT2A*; whole-exome sequencing; dual molecular diagnosis; eVai

1. Introduction

Shwachman-Diamond Syndrome (SDS) is an autosomal-recessive (AR) multi-systemic rare disease characterized by neutropenia, pancreatic exocrine insufficiency associated with steatorrhea and growth failure, skeletal dysplasia with short stature, and an increased risk of bone marrow aplasia or leukemic transformation. These are hallmark features that must be

fulfilled to meet the SDS diagnosis. Other aspects, including hepatic complications, cardiac involvements, endocrine dysfunctions, behavioral and cognitive function abnormalities, and ocular, dental, and dermal manifestations are also observed in patients [1–5].

Biallelic pathogenic variants in the *SBDS* gene are found in >90% of SDS patients [6]. Recently, pathogenic variants in *DNAJC21*(AR), *EFL1* (AR), and *SRP54* (AD) have been reported in SDS or SDS-like phenotypes in a few cases [7–12]. However, despite the fact that *SBDS* and *EFL1* mutations have been associated with SDS1 (OMIM #260400) and SDS2 (OMIM#617941), respectively, only *SRP54* has been associated with SDS-like conditions (OMIM#618752). Conversely, *DNAJC21* has been associated with bone marrow failure syndrome 3 (OMIM#617052).

The clinical spectrum of patients affected with SDS is wide [4]. Phenotypic variability has been noticed between patients, siblings, and even within the same patient over time, making clinical diagnosis challenging in some cases [13]. For instance, hematological abnormalities are extremely common and may range from neutropenia, found in 88–100% of patients, to hematological malignancies including myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML), which have been reported in about 17–35% of cases [13–16]. Pancreatic insufficiency is very common and found in 95% of patients. Additionally, failure to thrive, related to pancreatic insufficiency and feeding difficulties, has also been observed frequently (86.1%) [17]. On the other hand, skeletal defects, found in about 80.5% of SDS patients, are varied and may progress over time; they can also be widespread or localized, with variable degrees of severity [17].

Cardiac involvement, often reported as mild, is present in 43.5% of patients. Neuropsychosocial issues related to intellectual disabilities, learning difficulties, and psychomotor delay, have been reported in 20% of subjects [3,18]. Dermatologic issues have been found in 90% of SDS patients [17]. Furthermore, other features including renal abnormalities, dental problems, endocrine dysfunction, hepatic complications, ocular manifestations, and solid tumors have been observed in a few cases [19].

The clinical variation among patients has encouraged several researchers to study the genotype–phenotype correlation. Very recently, a genotype–phenotype study was conducted by Thompson et al. on a large cohort of SDS patients (74 patients, 42 families). The study revealed a narrow genotypic spectrum that was not significantly associated with the phenotype [17]. This work confirmed the results of previous studies [20]. Other studies have succeeded in explaining some features of the hematological variability through somatic genetic events. Two particular types of karyotype instability have been reported, including i(7)(q10) (the isochromosome of the long arm of chromosome 7) and del(20)(q) (interstitial deletions of the long arm of chromosome 20). Interestingly, these two clonal anomalies, in the absence of additional clonal chromosomal abnormalities, are associated with a lower risk of developing MDS and/or AML [21–23].

Modern genetic techniques such as the Next Generation Sequencing (NGS) have led to a broader and more comprehensive understanding of the genetic basis of a large set of diseases, from rare Mendelian disorders to hereditary cancer [24,25]. Nowadays, clinical laboratories have adopted NGS as the gold standard for the diagnosis of hereditary disorders because of its analytic accuracy, high throughput, and potential for cost-effectiveness [26,27].

In Mendelian diseases, NGS-based technology is considered a very strong tool, not only for the detection of pathogenic mutations [28], but also to explain phenotypic variability [29,30]. NGS technology, in addition to bioinformatics based on the automatic implementation of the ACMG guidelines and artificially intelligent variant interpreters, now allows the analysis of thousands of variants simultaneously for a single patient using a single assay [31].

In this paper, we present two SDS siblings, both carrying the same *SBDS* pathogenic variants and showing relevant differences in their clinical presentation. We demonstrate that part of the clinical variability can be explained using WES and bioinformatics tools.

2. Materials and Methods

2.1. Case Presentation

2.1.1. UPN42

This subject is a Caucasian female, and her weight at birth was 2870 g. In the first months of her life, she began to manifest a diarrheal alvus, and she was hospitalized several times for cough, fever, and dyspeptic symptoms, with features of pancreatic insufficiency. Since the first admission, a reduction in the number of neutrophils has been highlighted, and the hypothesis of autoimmune neutropenia was excluded. Gradually, over time, the symptoms of diarrhea with the characteristics of steatorrhea became more severe (5–7 discharges/day). At the age of 1 year, the hypothesis of cystic fibrosis was excluded via a sweat test, and SDS was diagnosed; oral replacement therapy with pancreatic extract was undertaken with good results. In regular follow-ups, up to the age of 21, the number of neutrophils was always lower than the average for her age. The hemoglobin values always remained within the normal range, and the count of reticulocytes was always appropriate for the hemoglobin levels. The platelet count was always lower than normal. Annual monitoring of bone marrow aspirates demonstrated poor or very poor cellularity, with notes of dyspoiesis in the myeloid precursors. The erythroid series in maturation was between 9 and 21% but it was not always observed; the share of mature lymphocytes reached a minimum of 44% and a maximum of 63%. Megakaryocytes were almost always absent. No blasts were found in any bone marrow examination and there was no evidence of any clonal evolution. She never requested blood transfusion or supportive therapy with G-CSF. At the age of 28, a FISH (Fluorescence In Situ Hybridization) examination confirmed the presence of a cell clone in 90% of the nuclei examined, containing a deletion of the centromeric region of chromosome 7 (7q11); this was consistent with the presence of i(7)(q10). At the age of 32, she developed Hodgkin lymphoma, and recovered after standard therapies; follow-ups are regular. The neuropsychological tests were normal, and the patient works as an employee.

2.1.2. UPN43

This subject is a Caucasian male, and the brother of UPN42. His weight at birth was 3450 g. An obstetric ultrasonography test revealed a horseshoe kidney with bilateral pyelectasis, more evident on the left kidney. At the age of two months, features of seborrheic eczema with a significant atopic component were found, and at the age of 2.5 months, the patient developed chickenpox. At the same time, a growth delay was evident (4900 g, <3%; length 58 cm, <3%; head circumference 39.5 cm, <3%), and the appearance of greasy and foul-smelling stools was reported by his parents. Chemical-microscopic examination of the stool showed evidence of abundant neutral acids and fats and a tryptic power <2.5 UT; the sweat test was negative, and molecular confirmation of SDS was obtained. Weight recovery took place progressively, adapting the diet. At the age of 2 years, the hypothesis of pancreatic insufficiency was confirmed, and oral replacement therapy with pancreatic extract and fat-soluble vitamin supplements was undertaken. Weight increase improved, and infectious episodes (bronchitis and persistent cough) were sporadic, similar to the episodes of steatorrhea. At the age of 6 years, irregularities in the metaphyseal regions of the long bones, particularly at the level of the proximal and distal ends of the femurs and of the proximal ends of the tibias, were demonstrated. The metaphyseal regions appeared enlarged, frayed, and unevenly thickened. The femurs appeared curved with a medial concavity, and the coxo-femoral joints were regular. Slight metaphyseal irregularities were also appreciable in the humerus, radius and ulna. The ribs were short and squat. At the same age, he underwent orchidopexy due to cryptorchidism.

The hematological examinations consistently showed leukocyte counts low for his age. The hemoglobin values always remained in the normal range, while platelet counts remained low. Regular bone marrow monitoring, starting at the age of 15, always demonstrated poor cellularity with notes of dysmyelopoiesis. The erythroid series varied between 9% and 25%. The proportion of mature lymphocytes was always between 38 and 53%. Megakaryocytes

have always been rare, and they were absent in the last check. No blasts have been found in any bone marrow examination, and no clonal evolution has been recorded. Similarly to his sister, he never requested blood transfusion or supportive therapy with G-CSF. FISH examination confirmed the presence of a cell clone in 90% of the nuclei examined, containing a deletion of the centromeric region of chromosome 7 (7q11), which is consistent with the presence of i(7)(q10). At last, minor facial dysmorphic features such as hypertelorism and wide nasal bridge were recorded. Records of family history show that he always needed additional support at school and that he can be defined as having a mild developmental delay, with problems in the areas of expressive language and memory; he has obtained a job within a national support program for people with minor handicaps.

2.2. Genetic Analysis

Genomic DNA was extracted from the peripheral blood of the two patients and their parents using the GenElute Blood Genomic kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. Whole-exome sequencing (WES) was performed as part of a project including 16 Italian SDS patients, using the HiSeq 1000 (Illumina, 2 × 100 bp). The patients, carrying biallelic pathogenic variants in the *SBDS* gene were identified by their Unique Patient Number (UPN), quoted in a previous study for our team [30]. Interesting variants found in the patients and their parents were validated using Sanger sequencing. Primers for variants of interest were designed using Primer3Plus (https://www.primer3plus.com/ 30 April 2022); PCR amplification was performed using a PrimeSTAR GXL DNA kit, and PCR products were purified using an enzymatic clean-up method (A'SAP-ArticZymes), followed by BigDye™ Terminator v3.1 Cycle Sequencing Kit. The samples were sequenced using a 3500 XL Series Genetic Analyzer.

2.3. Bioinformatics Analysis

2.3.1. The Human Phenotype Ontology (HPO) Terms

Based on clinical information for both patients, the Human Phenotype Ontology (HPO) database was used to select the HPO terms describing our patients' phenotypes (https://hpo.jax.org/app/ 22 April 2022) (Table 1).

Table 1. HPO [1] terms that describe UPN42 and UPN43 phenotypes.

UPN42	UPN43
HP:0001875 Neutropenia	HP:0000085 Horseshoe kidney
HP:0005528 Bone marrow hypocellularity	HP:0001875 Neutropenia
HP:0001738 Exocrine pancreatic insufficiency	HP:0005528 Bone marrow hypocellularity
HP:0001873 Thrombocytopenia	HP:0001738 Exocrine pancreatic insufficiency
HP:0040088 Abnormal lymphocyte count	HP:0003025 Metaphyseal irregularity
HP:0012189 Hodgkin lymphoma	HP:0012758 Neurodevelopmental delay
	HP:0001873 Thrombocytopenia
	HP:0000028 Cryptorchidism
	HP:0002719 Recurrent infection
	HP:0000431 Wide nasal bridge
	HP:0000316 Hypertelorism
	HP:0002474 Expressive-language delay

[1] HPO: Human Phenotype Ontology.

2.3.2. Filtering and Variant Prioritization

The Variant Call Format (VCF) files were generated via the ISAAC pipeline [32]; for data alignment and for variant calling, we used, respectively, the ISAAC aligner and ISAAC variant caller. We obtained a coverage mean of 65% and a coverage >20X corresponding to 91% of the complete exome sequences. For the annotation and the interpretation of the variants, we used enGenome eVai software (evai.engenome.com) which combines artificial intelligence with the American College of Medical Genetics (ACMG), AMP, and ClinGen guidelines to accurately report and classify genomic variants. Each genomic

variation is evaluated in terms of effect, filtered based on phenotypes (HPO), and a score of pathogenicity for every single genomic variant is provided [31,33].

By using eVai, two different types of filters were applied:

Filter (1): Variants with good quality (QUAL $\geq$ 30) and high pathogenicity score (pathogenicity score $\geq$ 3) were selected for both patients; then, shared variants were excluded.

Filter (2): Variants with good quality (QUAL $\geq$ 30) and that were related to each HPO term described in Table 1 were selected. Variants with low or no impact on gene function (pathogenicity Score < 0), and deep intronic variants were excluded.

2.3.3. Variant Interpretation

All variants resulting from filters 1 and 2 were screened according to their position, gnomAD frequency, mutation effect, mode of inheritance, mutation database data (ClinVar, LOVD, HGMD databases), literature, related diseases, and phenotypes using MalaCard, OMIM, and HPO. Additionally, in silico prediction tools including eVai pathogenicity score, ACMG, SIFT, MVP, FATHMM, Mutation Taster, M-CAP, and CADD were evaluated. For variants of interest, Uniprot (www.uniprot.org 10 January 2022) alignment tool was used to evaluate the amino acid conservation among different species such as humans, mice, rats, chickens, bovines, horses, and sheep.

The VCF file was also analyzed using DIVAs, an Explainable Artificial Intelligence (XAI) method for digenic variant identification and classification [34] (enGenome Srl). Briefly, this method, starting with patients' phenotypes (as HPO list) and variants (in VCF format), exploits features at the variant level, gene–gene interaction level, and phenotype level to classify the digenic combination's pathogenicity. Furthermore, XAI is used to investigate its digenic mechanism and subclassify each predicted pathogenic digenic combination as a True Digenic/Composite condition (whereby an interaction between the mutated genes triggers or exacerbates the phenotype), or a Dual Molecular Diagnosis (whereby two independent genetic events occur in the same individual, causing blended phenotypes).

3. Results

For both patients, the clinical diagnosis of SDS had been previously confirmed by the demonstration of two pathogenic variants in the *SBDS* genes c. [258 + 533_459 + 403del] and c. [258 + 2T > C], inherited from the father and the mother, respectively.

Using eVai, and according to the filter used, we selected:

Filter (1): 28 and 29 variants in UPN42 and UPN43, respectively.

(Supplementary Tables S1 and S2).

Filter (2): 14 variants in UPN42 and 82 variants in UPN43.

(Supplementary Tables S3 and S4).

All the variants were evaluated singularly, as mentioned in the Materials and Methods Section 2.3.3 (Variant Interpretation). eVai, of course, identified *SBDS* variants as relevant for patients' phenotypes.

For UPN42, the single variant analysis did not disclose any variation, which could be related to the patient phenotype. Conversely, a *KMT2A* variant (c.10663G > A, p.Gly3555Ser) was found to be related to some of the HPO terms that describe UPN43, including developmental delay, horseshoe kidney, bone abnormalities, cryptorchidism, expressive-language delay, and minor facial dysmorphisms of hypertelorism and a wide nasal bridge (Table 1). Pathogenic variants in the *KMT2A* gene cause Wiedemann–Steiner Syndrome (WDSTS, OMIM #605130), an autosomal dominant disorder. Interestingly, the phenotype of this syndrome includes all HPO terms selected for UPN43, which are absent in UPN42.

The variant was validated in the trio using Sanger sequencing and demonstrated to be de novo with a heterozygous status (Figure 1A).

The de novo variant c.10663G > A in *KMT2A* is a novel one; it is unpublished, and no allele frequency is reported. The variant is classified as likely pathogenic according to the ACMG guidelines, and classified as damaging according to SIFT, MVP, FATHMM, Mutation Taster, M-CAP, and CADD.

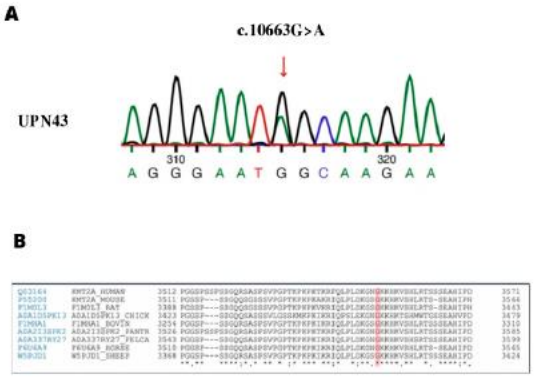

Figure 1. (**A**) Sanger sequencing confirmation. Part of the electropherogram illustrates the variant (c.10663G > A) in *KMT2A* in UPN43. (**B**) Uniprot alignment analysis shows that amino acid G (glycine) in position 3555 is highly conserved among different species such as humans, mice, rats, chickens, bovines, horses, and sheep.

Uniprot alignment analysis showed that amino acid G (glycine) in position 3555 is highly conserved among different species such as humans, mice, rats, chickens, bovines, horses, and sheep (Figure 1B).

As a further confirmation, the digenic combination involving *KMT2A* and *SBDS* genes in the UPN43 sample was predicted as pathogenic by DIVAs and subclassified as a likely Dual Molecular Diagnosis.

4. Discussion

Over the last decade, the introduction of WES and the implementation of Human Phenotype Ontology (HPO) has revolutionized the interaction between clinical diagnosis and molecular testing in monogenic and rare diseases. This combination allowed the time- and cost-effective sequencing of the whole exome to identify the genetic causes of a large number of syndromes with high clinical variability and genetic heterogeneity [35]. In medical genetic practice, WES has improved diagnostic yield by more than 25%, resulting in greater knowledge of the molecular mechanisms involved in Mendelian disorders [36].

SDS is a rare autosomal-recessive syndrome associated with three hallmark features including pancreatic insufficiency, neutropenia, and skeletal dysplasia. *SBDS* pathogenic variants are responsible for about 90% of reported cases [4]. However, the phenotypic spectrum of reported cases is wide, and the clinical variability among different patients, affected siblings, and in the same patient over time has been noted to be significant [17].

WES studies on SDS patients have been performed previously, either to identify new causative genes [8,10] or to explain the phenotypic variations found in some patients, including hematological problems [30,37,38].

Herein, we present two SDS siblings, (UPN42, female, and UPN43, male), who shared classical SDS features such as pancreatic insufficiency, hematological abnormalities (neutropenia, thrombocytopenia), and bone marrow hypocellularity. UPN43 showed additional characteristics not found in his sister, including skeletal problems (metaphyseal irregularities, metaphyseal-region enlargement, curved femurs, short ribs, and varus cervico-diaphyseal), renal abnormalities (horseshoe kidney with bilateral pyelectasis), recurrent infections, cryptorchidism, developmental delay, and minor dysmorphisms.

As an attempt to understand the clinical variability found in the two siblings, we analyzed WES data using the eVai and DIVAs platforms.

In UPN42, Hodgkin lymphoma (HL) was diagnosed; this disease has never been reported in any SDS patients, and the use of the eVai platform and DIVAs failed to identify any relevant variation, which is in keeping with the evidence of minor relevance of genetic factors in the development of HL. Recently, Weniger and a co-worker listed a number of

single-nucleotide variants found to be associated with HL in a limited number of cases. However, none of the variants listed were found in UPN42 [39].

In UPN43, in addition to *SBDS* mutations, we identified and validated a heterozygous de novo missense variant located in exon 27 of the *KMT2A* gene (Figure 1A). The variant is classified as likely pathogenic according to the ACMG guidelines, and its pathogenicity score of eVai is 5, which is considered a high score. As the variant (c.10663G > A, p. Gly3555Ser) in *KMT2A* was not reported before, no information related to its frequency is available; two pathogenic variants have been identified close to the site where our variant was found, within 100 bp [40].

KMT2A is located on chr11q23.3, consists of 37 exons, and encodes a DNA-binding protein that methylates a lysine residue on histone H3 (H3K4) [41]. According to mouse studies, *KMT2A* is abundantly expressed in adult hippocampus neurons and is required for synaptic plasticity, cognition, complex behaviors, and long-term memory [42,43]. Pathogenic variants in *KMT2A* cause chromatin-remodeling deficiencies, which lead to widespread alterations in gene expression throughout development, resulting in abnormalities in multiple body systems [44]. The reported variants are located quite uniformly through the sequence of the gene, and most variants are clustered in exon 27, which is compatible with the fact that it is the longest exon [40,45].

Germline mutations in *KMT2A* cause autosomal-dominant Wiedemann–Steiner Syndrome (WDSTS, OMIM #605130), and, in the vast majority of cases, de novo mutations have been confirmed [46]. Since the initial association between WDSTS and *KMT2A* [47], more than 250 sequence variations have been described [45,47].

WDSTS is an exceptionally rare, <1/1,000,000, chromatinopathy disorder characterized mainly by distinctive facial dysmorphism, hypertrichosis cubiti, developmental delay, skeletal anomalies, short stature, psychomotor delay, horseshoe kidney, and ocular, cardiac, and dental manifestations [40,48,49].

The phenotypic spectrum of the disease is very wide; extensive clinical variability has been reported [46,47], and is further expanded through the finding of more cases via WGS and WES [50]. The WDSTS genotype–phenotype correlation is currently not fully understood, and the mild/unusual WDSTS presentations may be challenging to recognize [51].

Intellectual disability/psychomotor delay, usually mild-to-moderate, has been reported in 65–100% of cases. The prevalence of autism has been estimated to be 11.8%, but subjects without autism may also reveal behavioral abnormalities such as ADHD, anxiety, and emotional dysregulation [52].

Facial dysmorphisms are common (50–70%), and differ from one patient to another; they include hypertelorism, long and downslanting palpebral fissures, long eyelashes, a wide nasal bridge, low-set ears, a thin vermilion, micrognathia, and anomalies of the dentition [53,54]. Skeletal abnormalities such as hip dysplasia, delayed bone maturation, a short palm, fifth-finger clinodactyly, and small and buffy hands have been found in about half of patients. Hypertrichosis cubiti has been reported in approximately 60% of cases [39,54].

The wide clinical spectrum also includes a significantly higher risk of developing recurrent infections [55], congenital heart disorders (in 30% of cases, including septal defect and patent ductus arteriosus), ocular manifestations (such as ptosis, squint, lacrimal duct anomalies, and refractive errors) in about 50% of cases. [56]. Renal anomalies, including horseshoe kidney, pyelectasis, small or hypoplastic kidneys, and cryptorchidism, have been reported in about 30% of cases [40,45,49,57].

If we compare the phenotype of UPN43 with the classical SDS description and WDSTS, we note that hematological problems and pancreatic insufficiency are SDS-related and not reported in WDSTS.

Developmental delay, of variable severity, is found in about 65–76% of SDS cases [58], so it is part of the typical description of the syndrome. It is also reported in 65–100% of WDSTS patients [46,59]. At present, as the percentages of patients with developmental

delay are roughly similar in both syndromes, so we cannot assign or define the specific contribution of each gene to this problem.

Although skeletal abnormalities are reported in about 50% of patients with WDSTS, the skeletal features of metaphyseal irregularities, curved femurs, and short ribs, present in UPN43, are considered classical for patients with *SBDS* mutations and more compatible with SDS than WDSTS. In the latter syndrome, the typical skeletal abnormalities are hip dysplasia, delayed bone maturation, and short palm and fifth-finger clinodactyly [40,59].

Recurrent infection episodes, present in UPN43, are typical for SDS as a consequence of neutropenia, which is mild-to-moderate, on different occasions. Infections have been reported in both syndromes, and we have no evidence, at present, of any interaction between *SBDS* and *KMT2A*, which could be related to the worsening of infections.

Conversely, horseshoe kidney with/without pyelectasis was never reported in any SDS patient (PubMed and Google Scholar search, June 2022, using horseshoe kidney AND Shwachman-Diamond Syndrome), and is found in approximately 0.25% of the general population; moreover, it is more commonly reported in WDSTS, with six patients showing this malformation [40,49,59–61].

Similarly, cryptorchidism, not reported in patients with *SBDS* mutations (PubMed and Google Scholar search, June 2022, using cryptorchidism AND Shwachman-Diamond Syndrome), is present in UPN 43, its prevalence in the normal population is 1.7%, and observed in about 20–35% of WDSTS cases [49,61,62]. However, despite the fact that cryptorchidism has been reported in some WDSTS patients, it has never been associated with *KMT2A* mutations; instead, it has been associated with *TASP1*, which is a regulator of *KMT2A* [63].

The features of facial dysmorphisms, horseshoe kidneys, pyelectasis, and cryptorchidism are not hallmarks of SDS; instead, they are more likely related to WDSTS. Therefore, we postulate that the *KMT2A* variant, found in UPN43, for which there is in silico evidence of it being pathogenic, has a concomitant and co-occurring clinical effect. This dual molecular effect, supported by in silico prediction via the DIVAs tool, could help to understand some of the clinical variations found among the siblings.

KMT2A was previously known as MLL (Mixed-Lineage Leukemia) because of its recognition as a frequent target of somatic rearrangements in acute leukemia [64]. Until now, no patients with WDSTS were reported to develop any type of hematological malignancy, so there is no evidence that the mutation we found acts as a risk factor for UPN43.

5. Conclusions

The reporting of these cases underlines the need for extended genome analysis in patients affected with SDS showing unexpected or unusual phenotypes. The availability of deep genome analysis and artificial-intelligence-based bioinformatics tools suggest that the well-known clinical variability reported in Mendelian diseases could be partially explained by these techniques. These new data are likely, in the future, to also be relevant for personalized medicine and therapy in selected cases or groups of patients.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/genes13081314/s1, Table S1: variants with good quality (QUAL $\geq$ 30) and high pathogenicity score (pathogenicity score $\geq$ 3) found in UPN42; Table S2: variants with good quality (QUAL $\geq$ 30) and high pathogenicity score (pathogenicity score $\geq$ 3) found in UPN43; Table S3: variants with good quality (QUAL $\geq$ 30) and that are related to each HPO term described in Table 1 for UPN42; Table S4: variants with a good quality (QUAL $\geq$ 30) and that are related to each HPO term described in Table 1 for UPN43.

Author Contributions: Molecular investigation: I.T., S.F. and A.M.; bioinformatic work: F.D.P., S.Z., I.L. and I.T.; variant interpretation: I.T., A.M. and C.D.; methodology design: A.M., C.D., I.T., F.D.P., S.Z. and I.L.; clinical data: U.R. and M.C.; writing of the manuscript: I.T., A.M., C.D., U.R. and F.D.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: We gratefully acknowledge the financial support from AISS (Associazione Italiana Sindrome di Shwachman) to A.M. This research was supported by a grant from the Italian Ministry of Education, University and Research (MIUR) to the Department of Molecular Medicine of the University of Pavia under the initiative "Dipartimenti di Eccellenza (2018–2022). We give special thanks to the Italian Agency for Development and Cooperation (AICS), the Center of International Cooperation and Development (CICOPS) at the University of Pavia, and the Partnership for Knowledge (PfK) initiative for offering a Ph.D. scholarship in Translational Medicine to I.T. This work is part of the Ph.D. thesis of I.T.

Conflicts of Interest: F.D.P., S.Z., and I.L. are full employees of enGenome Srl. I.L. and S.Z. have shares in enGenome Srl. The authors declare no conflict of interest.

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