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COORDINATOR Prof. Lorenzo Cosmi

Genetic diagnosis of Common Variable Immunodeficiency using Whole-Exome Sequencing

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Doctoral Candidate Dr. Boaz Palterer

(signature

Supervisor Prof. Francesco Annunziato (signature)

Coordinator Prof. Lorenzo Cosmi Dottorato di Ricerca in Scienze Cliniche Dipartimento di Medicina Sperimentale e Clinica Il Coordinatore Prof. LORENZO COȘMI Years 2018/2020

Abstract

Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency (PID) in adulthood. CVID is clinically and genetically heterogeneous. Patients have an increased susceptibility to infections, as well as autoimmunity, autoinflammation, lymphoproliferation, atopy, and cancer. In the last decades, Next Generation Sequencing (NGS) revolutionized the field of genetics. In the current 2018 IUIS classification the number of molecularly defined immunodeficiency syndromes exceeded 450. Whole exome sequencing (WES) is widely considered an efficient and cost-effective approach to patients with PIDs.

This study aimed to recruit a cohort of CVID patients and characterize them clinically, immunologically, and genetically. Using WES, we identified candidate variants in 50% of patients, including common disease-associated variants in TACI and BAFF-R genes, and novel variants in CTLA-4, CARD11, NFKB1, NFKB2, CD40LG, PIK3CD, PTEN, and TCF3.

An unbiased approach like WES allows for unexpected discoveries. A pathogenic variant of CD40LG was found in a patient diagnosed with CVID.

We also showed that *in silico* Copy Number Variants (CNV) prediction from NGS data can improve the diagnostic yield of WES. In a patient we found a deletion encompassing CD28, CTLA-4, and ICOS, leading to CTLA-4 haploinsufficiency.

The discovery of the genetic defects of PID diseases contributed to our understanding of the immune system. Moreover, dissecting the underlying molecular mechanisms of CVID can improve clinical management. A genetic diagnosis can give relevant prognostic information, help tailor targeted therapies and it can inform about the heritability of the disease.

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1. Introduction

Primary immunodeficiencies (PID) are part of the growing field of Inborn Errors of Immunity (IEI), which are defects of the immune system sharing an increased susceptibility to infections, and often characterized also by autoimmune, autoinflammatory, atopic, lymphoproliferative or neoplastic manifestations[1].

Singularly IEIs are considered rare diseases. However, as a group, their prevalence in the population is much higher than previously thought, with estimates up to 1/1000. In the last decade, the spread of next-generation sequencing (NGS) lead to the collapse of sequencing times and costs and revolutionized the PID field[2]. The last International Union of Immunological Societies (IUIS) IEI classification, published in 2019, included 430 separate entities. It is divided into 10 categories: Severe combined immunodeficiencies (SCID) and combined immune deficiencies (CID), syndromic PID, predominantly antibody deficiencies (PAD), diseases of immunodysregulation, phagocytic defects, innate immunity defects, autoinflammatory disease, complement deficiencies, bone marrow failure syndromes and IEI phenocopies[3,4].

PADs are the most prevalent subgroup in the population and include the most common PIDs in adults: selective IgA deficiency (sIgAD) and Common Variable Immunodeficiency (CVID) [5]. They also include childhood-onset PIDs, such as agammaglobulinemia and transient hypogammaglobulinemia. All in all, PADs are characterized by a predominant defect in the production of immunoglobulins by B lymphocytes but can also include a varying degree of T cell dysfunction.

Except for few well-defined syndromes, like Bruton's Agammaglobulinemia, the molecular defects underlying PADs and especially CVID have been elusive for decades. The main factors limiting the genetic study of CVID were the clinical and genetic heterogeneity of the disease, and the complexity, time, and money required for sequencing.

The development of NGS, and especially of unbiased methods like Whole Exome Sequencing (WES) and Whole Genome Sequencing (WGS), enabled the discovery of many novel genetic mutations. WES is widely considered a cost-effective approach in patients with PIDs. In this study, we aimed to recruit a cohort of CVID patients and characterize them by in-depth immunophenotyping and WES.

1.1. History, classification, and diagnostic criteria

PIDs are a relatively young field of medicine. Before vaccinations, antibiotics, rehydration therapy, and microbiology diagnostics, the mortality and morbidity of infectious disease were widespread. The development of modern medical practices paved the way to the description of the first PIDs.

Few reports at the beginning of the 19th century described pathognomonic clinical syndromes, such as Ataxia-Telangiectasia syndrome and Severe Congenital Neutropenia, which were years later characterized as PIDs[6]. However, Colonel Ogden Bruton is attributed with the formal discovery of the first PID is 1952, Bruton's Agammaglobulinemia. He was expecting to find an elevated gamma fraction in the serum protein electrophoresis of a boy with a history of multiple cases of pneumonia. However, the patient turned out to have a flat gamma zone. The serendipitous discovery led him to hypothesize that the boy's cause of infection was the absence of immunoglobulins. He then proceeded to treat the boy with purified immunoglobulins[7].

CVID is the most common symptomatic PID in adulthood, however, it's clinically and genetically very heterogeneous. The term CVID was coined in 1971 as an exclusion diagnosis from other well-characterized immunodeficiency syndromes, with typical clinical features and a consistent Mendelian pattern of inheritance[5].

The definition of CVID has varied a lot over the years. The ESID/PAGID 1999 Criteria included for a "Probable" diagnosis reduced IgG and IgA, age of onset >2, absent vaccination response and/or absent isohemagglutinins, and exclusion of other defined causes of hypogammaglobulinemia[8]. In the following years, the criteria have been refined by recognizing the heterogeneity of the disease.

Ameratunga 2013 criteria included a few characteristic laboratory and histological abnormalities, the need for infectious clinical manifestations or their sequelae, and the presence of autoimmune and/or lymphoproliferative complications[9].

A small proportion of patients presents with opportunistic infections as evidence of a T cell defect, beyond the infections typically associated with hypogammaglobulinemia. Those patients have been called "Combined Immunodeficiency" (CID) or "Late-onset Combined Immunodeficiency" (LOCID).[10] The 2014 revised ESID registry CVID criteria excluded LOCID patients.[11]

Regardless of the criteria used, the hallmark for the diagnosis of CVID is hypogammaglobulinemia. CVID's hypogammaglobulinemia is generally defined as low serum

IgG and the reduction of another immunoglobulin class, usually IgA and sometimes IgM. An important point to consider is that serum IgG levels are influenced by age and their distribution range is physiologically wide. IgG deficiency is commonly defined by total IgG values lower than two standard deviations from the mean in the age reference group.

Defective specific antibody production is another hallmark of CVID. Both T-cell dependent and T-cell independent responses should be evaluated. Specific post-vaccination titers can be used to monitor antibody responses. For T-dependent responses, the most used vaccines are tetanus and diphtheria, but also measles, mumps, and rubella vaccines can be used. The T-independent immune response is generally evaluated using the unconjugated pneumococcal polysaccharide vaccine, comparing levels before and one month after vaccination. Testing for isohemagglutinins is another strategy for testing anti-carbohydrate antibody production.

The onset of CVID might be at any age, but it's more often diagnosed in young adults between the ages of 15-40. While the symptoms can appear in early childhood, CVID is usually not diagnosed before the age of 3 years old, as in this age group it is recommended to search for alternative diagnoses such as transient hypogammaglobulinemia or other syndromic PIDs. On the other hand, due to the clinical heterogeneity, subtler presentations can suffer from a significant diagnostic delay.

The diagnosis of CVID requires to carefully exclude other causes of primary or secondary immunodeficiency. Many other defined PIDs can present with hypogammaglobulinemia. Moreover, it has become increasingly clear that CVID is an umbrella diagnosis and that several recently identified monogenic defects present as CVID-like disorders but are distinct entities.

1.2. Differential diagnosis

1.2.1. Secondary hypogammaglobulinemia

Hypogammaglobulinemia can be primary or secondary. The mechanisms leading to secondary hypogammaglobulinemia include defective production, excessive loss, or increased catabolism. Among primary forms, CVID is the most common cause, especially in adults, but many other PIDs can present with hypogammaglobulinemia and mimic CVID.

Secondary hypogammaglobulinemia is increasingly documented especially drug-induced hypogammaglobulinemia. Among the drugs most often involved there are chronic and high-dose corticosteroids, immunosuppressive therapies (mycophenolate mofetil, sulfasalazine, hydroxychloroquine, methotrexate), chemotherapy agents (cyclophosphamide, ibrutinib, imatinib), antiepileptics (valproate, phenytoin, carbamazepine, lamotrigine) and others (fenclofenac, captopril, etc.). An emerging common cause of secondary drug-induced

hypogammaglobulinemia is B cell depleting therapies, such as rituximab or ocrelizumab. In the last decade, the use of these drugs has considerably increased, from B cell lymphomas to many autoimmune diseases, such as multiple sclerosis, autoimmune cytopenias, myasthenia gravis, idiopathic inflammatory myopathies, and systemic lupus erythematosus[12].

Protein loss through urinary excretion (nephrotic syndrome) or gut loss (intestinal lymphangiectasias) can lead to hypogammaglobulinemia. Often those patients have disproportionally mild infections despite extremely low serum IgG values. Myotonic dystrophy type II, also known as proximal myotonic myopathy (PROMM), caused by CCTG expansion in ZNF9/CNTB exon 1, has an accelerated serum immunoglobulins catabolism and can therefore present with hypogammaglobulinemia[13].

Malignancies, especially hematological, are a major cause of hypogammaglobulinemia. Clonal B and plasma cell diseases are the most common, for example, Chronic lymphocytic leukemia, non-Hodgkin lymphomas, and multiple myeloma. Low-grade lymphomas can be challenging to diagnose and mimic CVID, especially in older patients[14]. CVID patients have usually normal or mildly reduced circulating B cell counts. B cell lymphocytosis should alert to the presence of a clonal disease or persistent polyclonal B cell lymphocytosis (PPBL), which can also cause hypogammaglobulinemia[15]. Good's syndrome is a rare cause of acquired adult-onset agammaglobulinemia in the context of thymoma[16].

Other causes of secondary hypogammaglobulinemia are a variety of infections, especially HIV, rubella, CMV, EBV, and toxoplasmosis.

1.2.2. Other Immunodeficiencies

The presentation of several PIDs can be indistinguishable from CVID, and the exclusion of other syndromes is especially important in childhood, as it can drastically change the prognosis and management. Moreover, with better access to sequencing, it is increasingly recognized how hypomorphic variants and atypical presentations of well-established syndromes can mimic CVID or other PAD.

Agammaglobulinemia is the prototypical PAD, the main difference from CVID is that circulating B cells are extremely low or virtually absent (<1%) and the immunoglobulin reduction is usually profound and involves all immunoglobulins classes. Most agammaglobulinemia patients are male children, as the X-linked form caused by defects in BTK is the most common cause, accounting for 85% of the patients. Biallelic defects of the BCR complex, assembly, and signaling (IGHM, CD79A, CD79B, IGLL1, BLNK) cause autosomal recessive agammaglobulinemia[17]. Interestingly, a complete deficiency of the regulatory subunit p85α of the PI3K (PIK3R1) causes

autosomal recessive agammaglobulinemia[18], while heterozygous defects cause Activated p110 δ syndrome (APDS) which is a CVID-like disease[19]. Autosomal dominant transmission was suggested due to mutations in LRRC8A after a patient with a truncation from a reciprocal translocation was found[20]. Both autosomal dominant and recessive forms of E47/TCF3 deficiency were associated with agammaglobulinemia[21,22]. One patient with agammaglobulinemia with a homozygous splicing variant in IRF4 caused by uniparental isodisomy was reported[23]. Biallelic mutation in the zinc transporter ZIP7, encoded by the SLC39A7 gene were reported in five unrelated families with agammaglobulinemia[24]. In 2001 Goldman et al. reported a monoallelic mutation in IKAROS, encoded by the IKZF1 gene, in an infant with agammaglobulinemia[25]. However, the phenotype of IKAROS deficiency was considerably expanded in recent years to include hypogammaglobulinemia, CVID-like disorders, CID, and an increased risk of leukemias[26]. Recently patients with autosomal dominant agammaglobulinemia facial dysmorphism and limb anomalies, previously described as Hoffman syndrome, were found to have mutations in TOP2B, encoding DNA topoisomerase 2-beta. These variants were found to be dominant-negative and causing defective proliferation, survival of pre-B-II cells and resulting in a block of B cell development[27].

Hyper-IgM syndrome was originally characterized as a marked deficiency in class-switched isotypes (IgG, IgA, IgE), with elevated polyclonal IgM. They have been therefore named class-switch recombination defects (CSRD)[28]. CSRDs are clinically and molecularly heterogeneous, ranging from severe multisystemic disease to mild PAD. The most common form is X-linked and caused by the deficiency of the CD40-ligand gene CD40LG, also known as CD154, expressed on activated T cells[29–31]. A much rarer autosomal recessive phenocopy is caused by biallelic mutations of the cognate receptor CD40, encoded by the TNFRSF5 gene, expressed on antigenpresenting cells[32]. As defects of CD40/CD40L impair T-B interaction, leading to a complex immunological dysfunction, they have been re-classified as CID. Similarly, ICOS deficiency was originally classified as a CVID-like disease, but it can present with a CSRD-like phenotype[33]. ICOS deficiency and the recently discovered ICOSL deficiency were also re-classified as CID[34]. Other CID characterized by T-B interaction defect can present with elevated IgM, like MHC-II deficiency.

Defects in the molecular machinery of class-switch recombination and somatic hypermutation (AID and UNG) and the DNA repair machinery involved can cause CSRD or syndromes with CSRDlike features. The most frequent autosomal recessive CSRD with a PAD phenotype is Activationinduced cytidine deaminase (AID) deficiency, caused by mutations in the AICDA gene[35]. Imai et al. described also autosomal dominant forms, presenting with a mild CSRD clinically mimicking CVID, caused by heterozygous AID mutations in the C-terminal nuclear export signal[36]. Deficiency of uracil N-glycosylase (UNG) and INO80 are very rare causes of autosomal recessive CSRD[37,38]. Class-switch recombination and somatic hypermutation introduce single-strand and double-strand DNA breaks, that are targeted by the Mismatch-repair (MMR) and the Non-Homologous End-Joining (NHEJ) molecular machinery. MMR defects such as PMS2 and MSH2/MSH6 can lead to CSRD-like phenotypes with familiar cancer predisposition[39,40]. NHEJ and DNA repair defects can also have CSRD-like features, Ataxia-Telangiectasia can present with elevated IgM, as well as hypomorphic Cernunnos and Ligase IV defects[41,42]. APDS, caused by gain-of-function mutations of PIK3CD or loss-of-function mutations of PIK3R1, can present with elevated IgM[43]. Finally, hypomorphic defects in NEMO, encoded by the IKBKG gene, can present with elevated IgM, mimicking a CSRD[44].

IgG subclass deficiency, IgA deficiency, and IgM deficiency

Immunoglobulins consist of two heavy and two light chains. There are nine heavy-chain classes and subclasses, μ (IgG), δ (IgD), α 1 and α 2 (IgA), γ 1, γ 2, γ 3 and γ 4 (IgG), ϵ (IgE), that pair with two light-chains κ and λ .

Heavy chain deficiencies are caused by deletions at the 14q32 locus. IGHM deletion completely impairs BCR signaling and B cell differentiation, leading to autosomal recessive agammaglobulinemia[17]. Deletion of other heavy chains can lead to IgG subclass deficiency, partial IgA deficiency, or IgE deficiency[45]. Very few cases of **light chain deficiencies** were reported, caused by deletion of the IGK or IGL loci. Patients can be identified by the absence of κ/λ bands with immunoelectrophoresis. Heterozygous deletions can be identified with flow cytometry of peripheral blood B cells finding a skewed Igk/Ig λ ratio[46].

Selective IgA deficiency (sIgAD) is the most common immunodeficiency, with prevalence up to 1:155. Most patients are asymptomatic, but some have an increased rate of even severe infections and complications such as bronchiectasis. There is also an association with atopy (food allergy, asthma) and autoimmune disease (systemic lupus erythematosus, atrophic gastritis, celiac disease, autoimmune thyroid disease, etc.)[47]. The genetic cause of sIgAD is unknown. The MHC haplotype HLA A1, B8, DR3, and DQ2 was shown to drastically increase the risk. There are familial cases of sIgAD and CVID segregating in the same kindred but without a defined Mendelian inheritance pattern[48]. The same rare variants in the TNFRSF13B gene, encoding TACI, have been associated with sIgAD and CVID[49]. GWAS studies identified IFIH1, CLEC16A, ICOS, CTLA4, and other genes, that have been also associated with CVID, as risk loci for sIgAD[50].

On the other hand, truly **selective IgM deficiency** is a rare and poorly defined entity, as many other acquired conditions, such as infections, autoimmune disease and malignancies, and other PIDs such as CARD11 deficiency, GATA2 deficiency, and 22q11 deletion syndrome, can cause decreased serum IgM. Moreover, no specific genetic defect has been identified. Therefore, it's unclear how many of the "IgM deficiencies" reported in the literature in the past are real selective IgM deficiency[51].

Total IgG is composed of the sum of the four IgG subclasses. The distribution of IgG subclasses is generally around 60-70% IgG1, 20-30% IgG2, 5-8% IgG3, and 1-3% IgG4. IgG1 and IgG3 can bind all Fcy receptors and activate the complement classical pathway. IgG1 and IgG3 are the most efficient mediators of ADCC and CDC effector functions against viral and bacterial antigens. IgG2 are weak complement activators, however, they can undergo covalent dimerization and form plasma IgM-IgG complexes. IgG2 are the predominant phagocytic antibodies recognizing polysaccharide antigens. IgG4 doesn't activate the complement and bind weakly Fcy receptors, they are regulated similarly to IgE and may form in-vivo bispecific antibodies Fab arm exchange. Because they don't cause receptors cross-linking and compete with IgE they are characterized as regulatory antibodies, especially in the context of IgE-mediated immunity[52].

IgG subclass deficiency is often asymptomatic, especially isolated IgG4 deficiency. Since IgG1 makes up two-thirds of the total IgG, IgG1 deficiency presents as classical hypogammaglobulinemia. IgG2 deficiency is usually associated with polysaccharide antigens responses[53], while IgG3 deficiency is associated with respiratory infections with viruses, *Streptococcus pyogenes*, and *Moraxella catarrhalis*[54]. IgG2/IgG4 and IgA deficiency are often associated, as well as IgG1 and IgG3. IgG Subclass deficiency, especially when associated with IgA deficiency, can evolve into CVID. Some well-characterized syndromes (Ataxia-Telangiectasia, Wiskott-Aldrich, etc.) can present with an associated IgG2/IgA deficiency.

Sometimes even with quantitatively normal classes and subclasses patients can display significant respiratory infections, possibly leading to bronchiectasis. **Specific antibody deficiency (SAD)** is defined by the failure to mount antibody responses to specific polysaccharide antigens, defective antibody response to protein antigens is usually associated with overt hypogammaglobulinemia or subclass deficiency. It is commonly tested by measuring antipneumococcal antibodies before and after the unconjugated 23-valent pneumococcal vaccine (PPV23). Some patients display a weak response (partial SAD), while others have a complete deficiency (total SAD), moreover, some patients can have normal responses but show a rapid decline of the antibody titers[55].

Transient hypogammaglobulinemia of infancy (THI) is characterized by low levels of IgG and/or IgA and IgM that spontaneously resolves within 4 years of age. It is considered a paraphysiological delay of the immune system development. Its prevalence is not exactly known, as probably most children are asymptomatic, but it's widely regarded as one of the most common hypogammaglobulinemia's differential diagnosis in early childhood[56].

Chromosomal abnormalities can also present with hypogammaglobulinemia[57]. Distal deletions of the long arm of chromosome 18 (18q syndrome) have been associated with IgA deficiency and have been shown to progress to CVID[58]. Deletions of 22q11 cause Di George syndrome, however, it was shown that milder cases with isolated hypogammaglobulinemia can be diagnosed in adulthood as CVID[59].

Defects of the epigenetic regulation lead to complex syndromes with relevant immune features[60]. Immunodeficiency centromeric instability and facial anomalies (ICF) syndrome is a rare autosomal recessive PID characterized by CVID-like hypogammaglobulinemia or agammaglobulinemia with B cells, typical facial anomalies, and mild developmental delay. More than half the cases are caused by a mutation in DNMT3B (ICF1), other genes that have been implicated are ZBTB24 (ICF2), CDCA7 (ICF3), and HELLS (ICF4). Regardless of the underlying molecular defect, ICF patients present with a similar phenotypical and immunological defect. They can be identified by screening for centromeric instability of chromosomes 1, 9, and 16 with a karyotype[61]. Kabuki syndrome is an autosomal dominant disease caused by heterozygous mutations of KMT2D or KDM6A. The most prominent features are intellectual disability, short stature, persistent fingertip pads, and skeletal abnormalities. The extent of the immunodeficiency was not widely characterized, few cases mimicking CVID have been described[62].

1.3. Clinical Presentation

Patients with CVID typically present with recurrent infections, especially of the airways, often complicated by bronchiectasis, and the gastrointestinal tract. While treatment of the infections is usually well managed by antibiotics and immunoglobulin prophylaxis, autoimmune and lymphoproliferative disorders are exceedingly common, and are the main prognostic and outcome predictors. The most prevalent non-infectious manifestations are autoimmune cytopenias, enteropathy, inflammatory bowel disease, granulomatous or lymphoid interstitial lung disease, nodular regenerative hyperplasia, or granulomatous disease of the liver, complications splenomegaly, and lymphoid hyperplasia. These often require immunosuppressive conundrum treatments, generating the clinical of using

immunosuppressive therapies in patients that are already immunocompromised. CVID patients can therefore be divided into two major clinical groups: patients with only infections (infection only CVID), and those complicated by non-infectious manifestations (complex CVID).

1.3.1. Infections

A history of recurrent bacterial upper and lower airway infections is the most common clinical presentation of hypogammaglobulinemia. Pneumonia is usually caused by common community pathogens, such as *Streptococcus pneumoniae*, *H. influenzae*, and mycoplasma species. Unlike the general population, those infections are more often severe, recurrent, requiring hospitalization and intravenous antibiotics. A significant percentage of patients develop acute complications, such as empyema, lung abscesses, sepsis, meningoencephalitis, osteomyelitis. Mycoplasma infections can lead to chronic destructive arthritis. Opportunistic pathogens such as *Pneumocystis jirovecii*, non-tubercular mycobacteria, fungal infections or CMV, should be an alarm for considering a more profound immunological defect. Chronic lung disease ultimately leads to the development of bronchiectasis, which represents a serious source of morbidity.

Gastrointestinal infections are also common in CVID, and they can cause acute or chronic diarrhea, which require a careful differential diagnosis from the autoimmune and lymphoproliferative enteropathies. *Giardia lamblia* is the most common pathogen, followed by *Campylobacter jejuni* and Salmonella species infections. *Campylobacter jejuni* can cause severe enteropathies mimicking IBD. Chronic Norovirus infections are an emerging cause of chronic enteropathy. While clearance of the virus is associated with amelioration of the villous atrophy, to date there is no recognized effective therapy. Infections with opportunistic pathogens like *Microsporidia, Cryptosporidia,* or other fungi and parasites, should prompt the research for CID. Cutaneous HPV infections, mucocutaneous candidiasis, saprophytic fungi infections and cutaneous leishmaniasis are usually seen in patients with CID[63].

1.3.2. Non-infectious complications

Around a quarter of patients with a PID will develop one or more autoimmune or inflammatory manifestations, therefore having a risk ranging between 3 and 14 times higher than the general population[64].

The most common autoimmune disease in patients with CVID and CID are cytopenias, in descending order idiopathic thrombocytopenia (ITP), autoimmune hemolytic anemia (AHA), and autoimmune neutropenia. The co-occurrence of ITP and AHA is named Evan's syndrome, and it can be often the disease presentation, especially in childhood[65].

Granulomatous disease in CVID is often misdiagnosed as sarcoidosis and can precede the overt hypogammaglobulinemia. Histologically the disease is characterized by well-defined noncaseating granulomas. The most affected organs are the lungs, lymph nodes, and spleen, but the involvement of the skin, liver, central nervous system, kidney, and gastrointestinal tract have been reported. Lung disease is called granulomatous interstitial lung disease (GLILD)[66]. Generally, no pathogens are isolated from this lesion. Although recently vaccine strain Rubella virus was isolated from the granulomatous lesions of some patients with CID[67].

Gastrointestinal involvement is one of the clinically most severe complications, presenting with chronic diarrhea and malabsorption. It can present with nodular lymphocytic hyperplasia, villous atrophy, or IBD-like pathology. The villous atrophy mimics coeliac disease, but it usually does not respond to an agglutinated diet. The IBD-like disease usually resembles Crohn's disease or microscopic colitis and can be associated with IBD comorbidities like biliary and hepatic disease[63].

Biliary and hepatic disease with cholestasis, primary biliary cirrhosis, and autoimmune or granulomatous hepatitis are potential causes of liver abnormalities. The most liver involvement is nodular regenerative hyperplasia, which may be indolent, but can lead to the serious complications of portal hypertension, such as cirrhosis and esophageal varices[68].

Organ-specific autoimmunity is common and includes thyroiditis, vitiligo, alopecia, atrophic gastritis, arthritis. Some more peculiar involvements like lymphocytic hypophysitis are rare, but more typically associated with some specific monogenic forms like CTLA-4 or NFkB2 deficiency[69].

Lymphoid hyperplasia can affect any organ. Splenomegaly is very common and can contribute to the cytopenias. Splenectomy or functional asplenia contribute to the immunodeficiency, especially to encapsulated bacteria, by impairing T-independent polysaccharide humoral responses. Massive lymphoproliferation in the mucosa-associated lymphoid tissues (MALT), in the airways (BALT) or in the gastrointestinal tract (GALT), can lead to obstruction as sometimes reported in patients with activated p110 δ syndrome. The differential diagnosis with malignant lymphoproliferative disease, especially with indolent lymphomas, can be difficult as clonality studies can be inconclusive and the pathology can be challenging. The research for EBV RNAs (EBER) should be always untaken to exclude EBV-associated lymphoproliferative disease. Autoimmune lymphoproliferative disease (ALPS) can also display several shared features and serum vitamin B12, soluble FasL, and circulating double negative (TCR $\alpha\beta$ +CD4-CD8-) T cells should be assessed[69].

Since the improvement of the mortality caused by infectious disease, following Ig replacement therapy and the antibiotic therapies, malignancies became the first cause of mortality in PID patients. Patients with CVID have an increased risk of developing cancers of all types estimated between 1.8 and 5-fold compared to the general population. Non-Hodgkin lymphoma and stomach cancer are the main offenders[70–72].

1.4. Lymphocyte subpopulations in CVID

Unsurprisingly, early efforts to dissect CVID heterogeneity investigated B lymphocytes [1]. Functional assays like B cell's mitogenic responses to various stimuli, like pokeweed, anti-IgM antibodies, or *Staphylococcus aureus*, showed that the B-cell defect in CIVD occurred at several levels, representing blocks at different stages of differentiation. [73,74] Bryant et al. classified CVID patients according to the secretion by B cells of IgM and IgG in response to anti-IgM and IL-2[75]. However, those functional assays were cumbersome and limited to the research setting.

The advent of flow cytometry played an instrumental role in the advancement of the understanding of B cell maturation and how it is impaired in primary immunodeficiencies. Moreover, it enabled the diffusion of classification methods that were easily accessible in the clinical setting.

Warnatz et al. in 2001 developed the **Freiburg classification**, identifying patients who had <0.4% switched memory B cells of total lymphocytes as the group I, while those with >0.5 were classified as group II. Subsequently, Group I was then subdivided into Ia and Ib according to the percentage of CD21 negative B cells, respectively over or under 20%. They also recognized that splenomegaly and autoimmune cytopenias clustered significantly in group Ia.[76]

Piqueras et al. in 2003 proposed a similar classification based on the expression of IgD and CD27, which was called the **Paris classification**. They identified three groups: MB2 with normal memory B cells; MB1 with defective switched memory but normal non-switched memory B cells (CD27+ B cell >11% but CD27+IgD- <8%) and MB0 with almost no memory B cells (<11% of B cells).[77]

In 2008, by combining aspects of both Freiburg and Paris classifications, a consensus was created based on a large multicentric European CVID cohort. The **EUROClass classification** confirmed the findings of the previous classifications. EUROClass created a separate category for patients with less than 1% of B cells of lymphocytes (B–). Then patients with B cells are divided into severe deficiency of class-switched memory B cells (<2% of CD19+ B cells, SmB–) and patients with more than 2% of class-switched memory B cells (SmB+). Between SmB- patients a small subgroup is

characterized by the expansion of transitional B cells (SmB–Trans high) and are significantly associated with lymphadenopathies. The expansion of the CD21low lymphocytes, in both SmB+ and SmB- classes, as found in the Freiburg classification, was associated with splenomegaly and autoimmune cytopenias[78].

Giovannetti et al. showed that many CVID patients displayed multiple **T cell abnormalities**, and those correlated with each other and were reflected in the loss of CD4+ naive T cells. They also demonstrated that those alterations correlated with the degree of B cell dysfunction measured as the percentage of switched memory B cells, and most importantly with the clinical severity[79].

Those important results were replicated in the large DEFI Study group cohort, enrolling over 300 CVID patients. In 2009 Malphettes et al. defined Late-onset Combined Immunodeficiency (LOCID) as a separate clinical entity from CVID, characterized by the occurrence of opportunistic infections and/or T CD4+ lymphopenia ($<200/\mu$ l). LOCID patients were more often children of consanguineous parents, displayed a higher prevalence of splenomegaly, granulomas, enteropathy, and malignancies, and required more antibiotic therapies and hospitalizations[10].

In 2014 the ESID CVID criteria were updated to exclude patients with CD4+ T cells < 200/µl and naïve (CD45RA+) CD4 T cells < 10%. The DEFI-LOCID criteria were later revised in 2015 to include patients with opportunistic infections and/or profound CD4+ lymphopenia (< 20/µl)[80]. In 2019 the Freiburg-CID criteria were proposed and included naïve (CD45RA+) CD4 T cells < 10% or a history of opportunistic infection[81].

T Follicular helper T (Tfh) cells are characterized by the expression of CXCR5 and are essential for GC formation and T cell-dependent humoral immunity. Dysregulation of Tfh function has been shown to contribute significantly to many immune disorders, such as autoimmunity, immunodeficiency, and malignancy. Studies have shown that circulating memory Tfh cells are composed of phenotypically and functionally distinct subsets. Similarly, to effector Th1, Th2, and Th17 cells, Tfh can be divided by the expression of chemokine receptors into type Tfh1 (CXCR3+CCR6-), Tfh2 (CXCR3-CCR6-), and Tfh17 (CXCR3-CCR6+). Activation can be measured by surface expression of PD-1, CCR7, and ICOS. Tfh2 promotes the secretion of IgG and IgE, while Tfh17 promote IgG and IgA responses. On the other hand, the help to class-switch recombination and Ig production by Tfh1 is less clear. For those reasons, Tfh2 and Tfh17 are regarded as "efficient helpers", while Tfh1 are not[82].

Defects in the frequency of Tfh and their subpopulations in monogenic PIDs have been systematically described by Ma et al. and showed how specific mutations affect the quantity and

quality of Tfh cells. They found that STAT3 LOF, IL10R, CD40LG, NEMO, ICOS, and BTK defects reduced Tfh cell frequencies, while STAT3 GOF, STAT1 GOF, and IL21/IL21R deficiency skewed Tfh cell differentiation toward a phenotype characterized by overexpression of IFN_γ and PD1[83]. These results underlined the importance of IFN_γ in the regulation of Tfh functionality and B cell homeostasis.

The analysis of the transcriptome of CVID patients with and without autoimmune complications, compared to agammaglobulinemia patients and controls revealed that there is a strong correlation between the complicated phenotypes and an IFN signature[84].

Collectively, these studies laid the foundation for the works that characterized Tfh and their subpopulation and function in CVID. First, it was shown that patients with CVID with autoimmunity or granulomatous disease had higher Tfh than patients with infection-only phenotypes[85]. Further characterization defined that Tfh2 and Tfh17 subpopulations were significantly decreased in CVID as compared with controls[86] and that Tfh from SmB- are skewed towards a Tfh1 phenotype[87]. Finally, it was shown that patients with immunodysregulation characterized by expanded B CD21lo cells had a skewed Th1 and Tfh1 phenotype from blood and lymph nodes, with increased IFNγ accompanied by a poor germinal center output and accumulation of Tbet+CD21lo B cells[88].

1.5. Genetic diagnostics in Primary Immunodeficiency and CVID

The first identified genetic defects in PID were discovered using Sanger sequencing in candidate genes and mapping large kindreds with clear mendelian patterns of inheritance. The identification of a causative genetic mutation is the ultimate diagnostic tool. However, univocal phenotype-genotype associations are rare for PIDs. It became increasingly evident how variants in several genes can be associated with the same or very similar phenotypes ("genetic heterogeneity"), on the other hand, one gene can be associated with many different phenotypes ("genetic pleiotropy"). Moreover, Sanger sequencing enabled exploring only one gene at a time and only pre-chosen candidate genes, stifling the opportunity for novel discoveries. For this reason, Sanger sequencing became rapidly too expansive and laborious, albeit remaining the sequencing gold standard for confirmation of mutations due to its high accuracy.

The development of Next Generation Sequencing (**NGS**) brought a genomic revolution to the field by providing high-throughput sequencing at a fraction of the cost and time per base sequenced. It enabled unbiased approaches like whole-exome and whole-genome sequencing, resulting in a burst in novel causal gene discoveries.

There are three NGS strategies commonly used: targeted gene panels (TGP), whole-exome sequencing (WES), and whole-genome sequencing (WGS). Each of these approaches have advantages and disadvantages which need to be weighed.

TGPs include a limited number of genes which is not appropriate for novel gene discovery. However, the limited number of targets enables better sequencing quality in terms of depth and coverage, lower costs, and often faster turnaround times.

WES is an extreme version of a TGP including all the protein-coding sequences (exons) encompassing around 2% of the whole genome. Since most disease-causing mutations are predicted to lay in protein-coding sequences, currently WES is considered the most cost-effective approach. Nevertheless, WES presents several limitations: it cannot identify mutations in non-coding sequences like deep intronic or regulatory regions, it's not suited for finding structural variants and it can have a hard time sequencing complex genetic loci containing pseudogenes, highly repetitive regions, and guanosine-cytosine (GC)-rich regions. Moreover, the analysis of the data can become challenging, as thousands of variants will be identified in every subject. Sifting between them requires complex filtering strategies, leveraging several bioinformatics tools to identify a needle in a haystack.

The current limitations of **WGS** are that it generates big amounts of data which are challenging to store and manage computationally, and the costs are on average three times higher than for WES. Although, WGS has some benefits over TGP and WGS. First, the lack of capture and PCRenrichment step leads to a more uniform sequencing depth and coverage and avoids GCamplification bias. Second, regulatory and intronic regions are sequenced. This allows identifying potential pathogenic non-coding variants and the identification of structural and copy number variants.

Copy number variation (**CNV**) are structural variants, larger than SNP and Indel variants, usually spanning over 1 kilobase, they can be deletions or duplications, and they are frequently involved in genetic diseases. They are not conventionally detected by NGS panels and WES, as most CNVs do not start or end within exons. For this reason, WGS or microarray-based comparative genome hybridization (array-CGH) are the preferred methods to identify structural variants and CNVs. However, over the last years, programs to call CNV from WES data have been developed[89,90].

The major challenge of WES, and even more of WGS, is variants interpretation. A single WES will typically produce between 50.000 and 100.000 variants, most of which are benign and common. To identify the candidate variants, they are annotated with several tools and filtered

sequentially. Finally, the variant's probability of being pathogenic can be classified according to the available data.

The bioinformatic pipeline processing the raw data into a variant file can be broken into three main parts: reads alignment, variant calling, and variant annotation. The raw sequencing data is stored in FASTQ files containing the single reads with quality annotations, those are processed and aligned to a reference genome with tools like Bowtie2[91] and BWA-MEM[92]. The most used human genome assemblies are the GRCh37 (hg19) and GRCh38 (hg38). The aligned reads are stored in a BAM file, those can the visualized in GenomeBrowser[93]. BAM files are then processed into VCF (variant call format) files by variant callers, such as GATK[94], SAMTools[95], FreeBayes[96]. VCF files contain the genomic coordinates of the variants (CHROM, POS), their dbSNP id (ID), the reference allele and the variant (ALT, REF), and more optional fields holding annotation information. During the annotation process information about the allele frequencies, biological data at the gene level, and the mutation level are retrieved and computed by dedicated software like VcfAnno[97], SnpEff[98], or ANNOVAR[99]. The annotated VCF files are then ready to be filtered by dedicated tools, such as GEMINI[100], VCF-Miner[101], BrowseVCF[102], VarAFT[103], or simply as excel tables.

The variant filtering strategy can be divided into sequential steps. In the first step, low-quality calls are removed. It is possible to remove variants contained in a pre-generated blacklist containing known cohort-specific false signals generated by incomplete reference genome assembly, location in low-complexity regions, bioinformatic misprocessing, or limitations inherent to cohort-specific private alleles[104].

Then only rare variants are selected. The variant frequency is compared to the general population using public large population databases, like GnomAD and 1000 Genomes[105]. Allele frequencies can vary dramatically in some populations because of genetic drift and founder effect, therefore dedicated databases of underrepresented ethnicities should be used when available. Many laboratories will create an internal reference database for their population. Variants with an allele frequency <1% are considered rare, however, based on the frequency of the disease and the inheritance pattern this threshold should be adjusted. For example, some known pathogenic variants like TNFRSF13B p.A181E have an allelic frequency of 2% in the Finnish population. The prevalence and the inheritance pattern permit to estimate the upper bounds of frequency for a pathogenic variant. Nevertheless, those assumptions should be taken carefully as hypomorphic variants and partial penetrance can yield unexpected results.

Variants can also be cross-referenced to databases containing known pathogenic variants, like HGMD[106] and ClinVar[107] to immediately identify already known pathogenic mutations.

Variant-level and gene-level criteria are used to further filter the data. Many variant-level annotations can be used, starting with the prediction of the variant function location and effect. Exonic and splicing variants are better covered in WES and more probably pathogenic, compared to 5' and 3'UTR, ncRNA, intronic and intergenic regions. Nonsense variants, splice site mutations, frameshift mutations, and mutations affecting stop and start codons are more likely to be pathogenic. The most common SNP are synonymous mutations and are generally considered to be benign, but in rare cases, they can affect splicing and RNA stability. Missense mutations are the most challenging to assess because of how unpredictable the substitution of a single amino acid can be on a protein function. Many scores have been developed to predict missense variants deleteriousness. Sorting Intolerant from Tolerant (SIFT) by using sequence homology predicts the effects of all possible substitutions at each position in the protein sequence[108]. Polymorphism phenotyping v2 (PolyPhen2) predicts the possible impact of amino acid substitutions on the stability and function of human proteins using structural and comparative evolutionary considerations[109]. Many scores leveraging different features have been developed, such as conservation (phastCons, PhyloP, GERP), epigenetic modifications (H3K4Me1, H3K9Ac, DNase-Seq), functional prediction (amino acid change, TF motif disruption), genetic context (GC content, CpG content, transversions). This large number of annotations are often combined in integrative scores, trained over big datasets with machine learning techniques. Combined Annotation-Dependent Depletion (CADD) is an integrative annotation built from more than 60 genomic features widely used for scoring missense and indel variants[110]. DANN uses the same feature set and training data as CADD to train a deep neural network (DNN) achieving better sensitivity and specificity[111]. Many such scores have been developed in recent years, like FATHMM[112], REVEL[113], and BayesDel[114].

Gene-level annotations are especially useful when looking at all genes and not only at a candidate gene panel. Tools like the Human Gene Connectome (HGC) and the Closest Disease-causing Genes (CDG) are useful to prioritize genes that interact or are in the same pathway as known pathogenic genes. They are based on protein-protein interaction databases, known pathogenic variants databases (HGMD, OMIM) and they also leverage phenotype information from the human phenotype ontology (HPO).

An important piece of information is the gene expression in human tissues and animal models. Patients with immunodeficiency are expected to harbor their pathogenic variants in genes that

are expressed in the immune system cells. This can be evaluated using transcriptome databases like GTEx[115], which compares gene expression across several human tissue and organs, or immune system-specific databases like DICE[116]. Recently variants in non-immune cells have also been associated with immunodeficiency disease, mostly by affecting intrinsic innate immunity[117]. The presence of a relevant mouse phenotype can be evaluated by searching the Mouse Genome Informatics (MGI) database[118].

Population genetics show the evolutionary constraint and purifying selection a gene is under. The probability of being loss-of-function intolerant (pLI), and similar metrics, are widely used. It classifies genes into null, recessive, or haploinsufficient, based on the number of observed vs expected loss-of-function variants[119]. DOMINO is a tool assessing the likelihood for a gene to harbor dominant changes, based on a machine-learning approach to extract discriminant information from a broad array of gene-specific features[120].

Gene Damage Index (GDI) is a genome-wide, gene-level metric of the mutational damage that has accumulated in the general population. A small percentage of human genes account for a great number of variants, these genes are under lower evolutionary pressure because they are more likely redundant and/or non-essential. Therefore, a high GDI implies a lower likelihood of harboring pathogenic variants[121].

Additionally, gene-levels annotations can improve variant level scores by putting them into a context. For example, the Mutation Significance Cutoff (MSC) defines gene-specific significance cutoffs for SIFT, PolyPhen2, and CADD scores[122].

Knowledge of the clinical and immunological phenotype of the patient and the medical history of his family is essential to formulate a sound genetic hypothesis. Having access to the sequencing data of first-degree relatives of the patients, particularly the parents, ease exponentially the filtering process. A trio design was shown to improve the diagnostic yield for WES[123]. When both parents are unaffected, the researcher can prioritize homozygous, heterozygous compound, and de novo mutations. While in the case of autosomal dominant transmission, heterozygous variants inherited from the affected parent will be prioritized.

The identified variants are reported integrating all the above-discussed information. One of the most used platforms for variant reporting is the ACMG variant classification criteria. Variants are classified as 'pathogenic', 'likely pathogenic', 'uncertain significance', 'likely benign', and 'benign'. The process of classification is based on scoring five domains (e.g. population data, computational data, functional data, segregation data, etc.)[124].

Experimental validation of the causal relationship between genotype and phenotype is crucial, especially for a variant of uncertain significance (VUS) and likely pathogenic variants. Considering the high number of variants identified by NGS, many false-positive variants are generally identified. Therefore, a rigorous experimental approach to functionally validate the variants is always necessary. This can become very challenging when dealing with variants in genes with unknown functions. A guideline for the experimental evidence required to validate new variants proposed three criteria: i) the variant must segregate with the phenotype and not occur in healthy subjects, ii) experimental studies must show an altered function or expression of the gene product, for example, reduced expression or functional change in the pathway, iii) the phenotype must be replicated in a cellular model or an animal model by introducing the pathogenic variant or rescued by introducing the wild type allele[125].

1.6. Monogenic defect in CVID and CVID-like disorders

The first monogenic defect identified in CVID was the deficiency of the Inducible Co-stimulator (**ICOS**)[126]. ICOS is expressed by activated T cells and plays a central role in the T-B interaction. The ICOS/ICOSL complex along the CD40/CD40L mediates Tfh cells function and germinal center (GC) formation. Transmission is autosomal recessive, and patients have been identified in childhood or as adults[33]. ICOS deficiency characteristically causes a marked reduction of circulating Tfh cells (CD3⁺CD4⁺CD45R0⁺CXCR5⁺)[127]. In later years the ICOS deficiency disease spectrum was expanded demonstrating the combined nature of the defect. ICOS deficiency, along with the newly discovered **ICOSL** deficiency[34], has been re-classified as CID[4].

The **B cell co-receptor complex** is composed of CD19, CD81, CD21, and CD225[128]. Upon interaction between the BCR and the antigen, the help CD19 complex amplifies the signal through the PI3K and NFkB pathways.

Biallelic defects of **CD19** and **CD81** cause absent surface CD19 expression, and result in a severe reduction of serum IgG[129]. The complex is also engaged by the complement system as **CD21** is a receptor for the CD3d fragment. Defects of CD21 result in a milder phenotype[130].

CD20, encoded by the MS4A1 gene, is involved in the transmembrane Ca2+ transport. However, only a single patient was described, harboring a homozygous cryptic splicing mutation. Moreover, this patient did not fulfill CVID criteria[131].

The **TNF receptor superfamily (TNFRSF)** including B-cell activating factor receptor (BAFF-R, encoded by TNFRSF13C), B-cell maturation antigen (BCMA, encoded TNFRSF17) and Transmembrane Activator and Calcium modulator and CAML Interactor (TACI, encoded by TNFRSF13B), and their ligands B-cell activating factor (BAFF, encoded by TNFSF13B) and A

Proliferation-Inducing Ligand (APRIL, encoded by TNFSF13) are critical in multiple stages of B cell development and survival[132].

TACI mediates IgA and IgG CSR, differentiation and survival of plasma cells and development of T-independent polysaccharide responses. BAFF-R plays a role in peripheral B cell survival and maturation. BCMA promotes long-term plasma cell survival in the bone marrow.

Variants in **TACI are** the most frequent genetic alteration in CVID and they are found in around 10-15% of CVID patients. They are frequently associated with slgAD and other antibody defects[133]. Biallelic variants are often associated with some antibody deficiency. On the other hand, monoallelic variants of TNFRSF13B have been detected in healthy relatives of patients and the general population at a 1% frequency. Thus, those variants are likely disease-modifying rather than disease-causing[134].

Many TACI mutations have been described, including missense variants and deletions, and spanning the whole length of the gene. The most common are C104R and A181E, accounting for over 80% of the monoallelic variants. Those missense variants have been proposed to act as dominant-negative impairing B cell homeostasis[135]. Monoallelic, but not biallelic variants, have been shown to increase the risk of autoimmunity, possibly by damaging the removal of autoreactive B cells[136,137].

Patients with Smith-Magenis syndrome (SMS), caused by 17p11.2 deletions encompassing the TNFRSF13B gene, can display hypogammaglobulinemia[138]. Study of patients with SMS and patients carrying null variants of TACI, such as c.204insA, show that TNFRSF13B haploinsufficiency acts at later stages of B-cell development, without the autoimmunity features of the monoallelic missense variants.[139]

BAFF-R, encoded by the TNFRSF13C gene, has been reported in several kindreds with hypogammaglobulinemia and CVID. Most reported patients are either homozygous, heterozygous, or compound heterozygous for the two missense variants P21R and H159Y. While these variants are functionally relevant, acting respectively as a loss-of-function and a gain-of-function, it is still debated if they should be considered disease-causing or disease-predisposing[140–142]. The few patients with complete loss of BAFF-R that have been published showed a marked increase of transitional B cells[143]

Terminal differentiation of memory B cells to plasma cells is critically mediated by **APRIL**, encoded by TNFSF13. A homozygous frameshift mutation of TNFSF13 in a CVID patient of consanguineous parents was recently described. The patient had elevated marginal zone-like B

cells, but reduced plasma cells, confirming the role of APRIL in plasmablasts differentiation and immunoglobulin production[144].

A mono-allelic mutation in TNF-like weak inducer of apoptosis **(TWEAK)**, encoded by TNFSF12, was described in a single family with autosomal dominant CVID. The TWEAK variant R145C in the TNF-homology domain was shown to form high molecular weight aggregates with BAFF, resulting in a downregulation of BAFFR signaling and defective noncanonical NF-κB pathway[145].

A heterozygous mutation in Interferon Regulatory Factor-2 Binding Protein 2 (**IRF2BP2**) was found to segregate in a family with CVID (father and two children). The patients suffered from hypogammaglobulinemia and autoimmunity. *In vitro* studies demonstrated impaired B cell maturation, but the exact disease mechanism is unclear[146].

Cbl-interacting protein of 85 kD (CIN85), also known as SH3KBP1, links the BCR to the canonical NFkB pathway contributing to T cell-independent immune responses[147]. CIN85 gene is located on the X chromosome and patients with germline deletions were found to have an X-linked isolated B cell defect[148].

The enzyme tRNA nucleotidyltransferase, encoded by **TRNT1**, synthesizes CCA sequences at the 3' end of tRNA[149]. Biallelic mutations of TRNT1 were first described in Sideroblastic anemia, anemia, fevers, and developmental delay (SIFD)[150]. The phenotype was later expanded with adult-onset B cell deficiency[151,152], panniculitis[153], cataract and inner retinal dysfunction [154].

The **NF-kB family** includes 5 genes, RELA (p65), NFKB1 (p105/50), NFKB2 (p100/52), RELB, and c-REL. The canonical pathway is mediated by p65:p50 (RELA and cleaved NFkB1) heterodimers. It is downstream of the TCR/BCR and TLRs. The non-canonical pathway mediates the signaling of TNF superfamily receptors such as ICOS, TACI, BAFFR, BCMA, and CD40. NFkB2 is processed by the proteasome from p100 to p52 and binds to RelB.

Autosomal dominant mutations in **NFKB1** are the most common cause of CVID in Caucasians. While most of the mutations act as haploinsufficiency, some have different disease mechanisms. Several small cohorts and case reports described NFKB1 deficiency as a CVID-like disease with prominent autoinflammatory and autoimmunity features. The collection of large multicentric cohorts unveiled the heterogeneity of the disease. Strikingly, it was shown to have incomplete clinical penetrance (70%), with some patients presenting as CID, and a high frequency of autoimmunity, lymphoproliferation, enteropathy, opportunistic infections, autoinflammation, and malignancy[155,156]

Heterozygous mutations in **NFKB2** have been associated with a CVID-like disease characterized by early-onset and features of CID. Lymphocytic organ-infiltrations, enteropathy, arthritis, alopecia, cytopenias are common[157]. The pathognomonic features are endocrine abnormalities, particularly adrenocorticotropic hormone (ACTH) insufficiency, and other pituitary hormone deficiencies, which led to naming it Deficient Anterior pituitary with Variable Immune Deficiency (DAVID) syndrome[158].

The Phosphatidylinositol 3-Kinase (PI3Ks) are a family of enzymes involved in many key cellular functions, including metabolism control, growth, survival, and differentiation. There are four classes of PI3K: I, II, III, and IV. Class I PI3Ks catalyze in vivo the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Class I PI3K are heterodimeric and composed of one catalytic subunit and one regulatory subunit. The catalytic subunits are called p110, and there are four types: $p110\alpha$ (encoded by PIK3CA), $p110\beta$ (encoded by PIK3CB), p110y (encoded by PIK3CG), and p110 δ (encoded by PIK3CD). The regulatory subunits are p85 α and its splicing variants p55 α and p50 α (encoded by PIK3R1), p85 β (encoded by PIK3R2), p55y (encoded by PIK3R3), p150 (encoded by PIK3R4) and p101 (encoded by PIK3R5). While p110 α and p110 β are ubiquitously expressed, p110 δ is expressed primarily in lymphocytes [159]. In resting lymphocytes p110 δ is inhibited by p85 α . Upon stimulation of numerous receptors, the PI3K complex is recruited to the membrane and activated by the disengagement of the regulatory subunit. The generation of membrane PIP3 enables the docking of Pleckstrin homology (PH) domain-containing proteins, like AKT, PDK1, and Tec. Other enzymes such as PTEN and SHIP act as negative feedback by reconverting PIP3 to PIP2. Downstream of the PI3K activation there is a cascade of serine/threonine kinases, starting from AKT, mTOR, 4E-BP, and the S6K complex. This leads to increased glucose uptake and glycolysis, cell-growth, proliferation, and increased survival[159].

Heterozygous mutations in **PIK3CD** in patients with CVID were first reported by Jou et al. in 2006 [160]. It was only years later that the disease presentation and immunological phenotype were elucidated[161,162]. These variants were shown to be gain-of-function, resulting in overactivation of the catalytic p110 δ subunit. The disease was renamed Activated P110-Delta Syndrome (APDS because of the unchecked PI3K/Akt/mTOR activation leading to lymphoproliferation, immunosenescence, and lymphoma predisposition. Most patients were shown to harbor the missense mutation E1021K. The phenotype was broadened to include

presentations as CID and hyper-IgM, as many patients have elevated serum IgM. Typically, patients have elevated transitional B cells and elevated senescent (CD57+) terminal effector memory T CD8+. Also, biallelic LOF PIK3CD variants have been shown to cause a similar phenotype, demonstrating how both hyperactivity and deficiency of PI3K signaling is deleterious to the immune system homeostasis.[163–165]

Heterozygous splice-site mutations leading to skipping of exon 11 of **PIK3R1** generate a loss-offunction of p85 α regulatory subunit that is unable to properly regulate p100 δ activity, therefore resulting in a functional gain-of-function[19,166]. The disease is called APDS2 due to the similarity with the GOF variants of PIK3CD. Probably due to the broader role of PIK3R1, interacting also with the p110 α and p110 β , other non-immunological features like growth retardation, dysmorphism, and mild neurodevelopmental delay are significantly more frequent in APDS2 then in APDS1[167]. Notably, SHORT Syndrome (Short stature, Hyperextensibility of joints, Ocular depression, Rieger anomaly, and Teething delay) is caused by LOF PIK3R1 mutations. In the original descriptions of the SHORT syndrome, there was no immunological phenotype. However, further studies characterized the overlapping spectrum between SHORT syndrome and APDS2[168]. Biallelic LOF mutations of PIK3R1 are associated with agammaglobulinemia[18].

Mutations in the Phosphatase and Tensin homolog protein (**PTEN**) also leads to hyperactivation of the PI3Kδ complex, as seen in APDS1 and APDS2 and have been therefore named APDS-like (APDS-L)[169]. The immunological phenotype is generally milder, while other features prevail, such as solid tumor predisposition, macrocephaly, and developmental delay[170]. PTEN mutations have been comprehensively characterized as the cause of the hamartomatous diseases Cowden syndrome and Bannayan-Zonana syndrome or Ruvalcaba-Riley-Smith syndrome [171].

Besides PI3K activation to generate PIP3, TCR engagement also activates phospholipase C-γ to generate inositol 1,4,5-triphosphate (IP3) that activates store-operated Ca2+ entry (SOCE). The enzyme Inositol phosphate 3-kinase (**ITPKB**) converts IP3 to IP4, creating a positive feedback with ITK and dampening SOCE and AKT activation. One CVID patient harboring a microdeletion in 1q42.1-3 encompassing the ITPKB gene was described[172]. Another frameshift variant c.146_147insA of ITPKB was found in a patient in a cohort of PID with elevated serum IgMs[173]. A homozygous mutation of ITPKB was recently described in an infant with SCID[174].

ARHGEF1 is a RhoA-specific guanine nucleotide exchange factor expressed in hematopoietic cells. Biallelic ARHGEF1 mutations caused low RhoA activity, causing impaired actin

polymerization and AKT regulation. Both described patients had low marginal zone and memory B cells and increased transitional B cells[175].

Sec61 translocon alpha 1 subunit (SEC61A1) is part of the Sec61 polypeptide-conducting channel complex in the endoplasmic reticulum membrane. The Sec61 complex is induced during plasma cell differentiation, in preparation of the immunoglobulin production and secretion. SEC61A1 variants were found to segregate with CVID in two large families with an autosomal dominant disease. Interestingly, the patients had an infection-only CVID phenotype and displayed normal peripheral B and T subpopulation, but an intrinsic defect of PC differentiation. It was shown that the variant V85D triggered the terminal unfolded protein response in multiple myeloma cell lines, pinpointing a selective plasma cell survival defect.[176] Variants in SEC61A1 have also been associated with tubulointerstitial and glomerulocystic kidney disease with anemia[177] and severe congenital neutropenia[178]. Further studies are needed to elucidate the pathophysiology, the genotype-phenotype correlation, and the overall disease spectrum.

IKAROS zinc finger, encoded by the **IKZF1** gene, is a master regulator of hematopoiesis expressed in lymphoid cells. IKZF1 deficiency was first described in an infant with pancytopenia and agammaglobulinemia[25]. Two distinct phenotypes can be distinguished based on the mutation effect. Autosomal dominant mutations causing haploinsufficiency present as CVID[179,180], while dominant-negative mutations in the DNA binding domain are associated with CID with pancytopenia and opportunistic infections[26]. It was also observed that families harboring IKZF1 variants had a predisposition to childhood acute lymphoblastic leukemia[181].

RAC2 is a Rho GTPase regulating a variety of cellular processes of neutrophils and lymphocytes. The first described RAC2 variants were dominant-negative defects leading to a prominent neutrophil migration defect, characterized by a negative DHR after fMLP stimulation[182,183]. It was later noted that those patients also displayed adaptive immunity defects, a notion that was confirmed by the description of gain-of-function mutations with a phenotype ranging from SCID to CID[184–186]. A homozygous nonsense mutation was found in two siblings with a CVID, they had only a modest chemotactic and granule exocytosis defect[187].

Congenital disorders of glycosylation (CDG) are a vast group of rare syndromes that affect genes implicated in the glycosylation of proteins. Their clinical presentation is heterogeneous, and often including varying degrees of immunological dysfunction.

Mannosyl-oligosaccharide glucosidase, encoded by the **MOGS** gene, is an endoplasmic reticulum enzyme implicated in protein glycosylation. Mutations in MOGS lead to a syndrome characterized by dysmorphic facial features, hypotonia, seizures, developmental delay, cerebral

atrophy, and severe hypogammaglobulinemia. B cell development, maturation, and survival appear to be normal, but the produced immunoglobulins have a short half-life due to the glycosylation defect.[188]

Mutations in the vacuolar-type H+-ATPase subunit **ATP6AP1** also cause a CDG characterized by immunodeficiency, liver disease, *cutis laxa*, and psychomotor impairment. The described patients had T CD4 and CD8 lymphopenia, as well as hypogammaglobulinemia and poor polysaccharide vaccine responses.[189]

Recently a group of disorders that were originally discovered in CVID cohorts, and characterized by prominent autoimmunity and lymphoproliferation, were re-classified as **"disorders of Immunodysregulation"**. Nonetheless, their presentation can be indistinguishable from complicated CVID, but their identification is paramount, as in some cases targeted therapies are available.

Cytotoxic T lymphocyte antigen-4 (CTLA4) is a member of the CD28 receptor family. CTLA-4 acts as a negative regulator by competing with CD28 for the B7 family molecules, CD80 and CD86, expressed on antigen-presenting cells. However, most of the CTLA-4 inhibitory function is mediated by Treg, who expresses CTLA-4 at higher levels. Upon binding to CD80/CD86 the complex CTLA-4/B7-molecule is internalized by clathrin-mediated endocytosis and shuttled in a dynamin-dependent manner to the endosomes[190]. CTLA-4 interacts through his cytoplasmic tail with the Lipopolysaccharide-responsive beige-like anchor (LRBA) protein. LRBA protects CTLA-4 from lysosomal degradation[191]. Direct activation of RAB11 by DEF6, a GEF protein downstream of TCR signaling, mediates vesicular trafficking of internalized CTLA-4 molecules to recycling endosomes, the trans-Golgi network, and back to the cell surface[192].

Autosomal dominant **CTLA-4** deficiency has been identified in patients with immunodysregulation, characterized by variable levels of hypogammaglobulinemia and lymphocytic organ infiltration[193,194]. Both missense variants and mutations leading to haploinsufficiency cause the disease. The study of large kindreds demonstrated a variable penetrance (~70%), an unpredictable age of onset and clinical course, ranging from asymptomatic to life-threatening poly-autoimmunity even in the same family. The most common clinical manifestation are autoimmune cytopenias, enteropathy, GLILD, central nervous system lymphocytic infiltration. There is also an elevated risk for malignancies, in particular lymphomas and gastric cancer[195]. The immunological phenotype is characterized by T CD4+ lymphopenia, Tregs can be normal or even elevated but are functionally impaired, B cells are usually reduced with expanded CD21Io B cells[193,194].

Concurrently, autosomal recessive defects in **LRBA** were described in patients with very similar clinical manifestations to CTLA-4 deficiency. LRBA deficiency is more severe, with an early age of onset and complete penetrance[196,197]. The clinical presentations of LRBA and CTLA-4 deficiency can overlap ALPS or IPEX. Treatment with abatacept, a recombinant fusion-protein Ig-CTLA-4, or sirolimus, an mTOR inhibitor, has shown promising results in the management of CTLA-4 and LRBA deficiency. However, some patients might not respond fully and the risk for immunosuppression, especially causing EBV/CMV reactions, must be taken into consideration.[198,199] Because of the relevant disease burden, especially in young LRBA patients, there is mounting evidence for treating with HSCT[195,200].

Recently 7 patients across 3 kindreds with homozygous mutations in **DEF6** were identified presenting with autoimmunity, lymphoma, and EBV lymphoproliferation. Patient's cells exhibit impaired regulation of CTLA-4 surface trafficking and availability[192,201].

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways are central in the immune system homeostasis. JAK-STAT signaling is downstream of cytokine receptors, and it plays a pivotal role in T helper cell polarization. This pathway is regulated by several regulator proteins, including Suppressors of Cytokine Signaling (SOCS), Protein Inhibitors of Activated STATs (PIAS), and Protein Tyrosine Phosphatases (PTPs). Several immunodysregulation syndromes have been associated with different functional variants, demonstrating the sensitivity of this pathway to fine-tuning[202].

STAT3 is part of the downstream signaling of interferons, IL-6, and IL-21 receptors. LOF mutations in STAT3 are the cause of the classic autosomal dominant Hyper-IgE Syndrome. More recently GOF mutations have been reported in an autosomal dominant disease with autoimmunity, immunodeficiency, and lymphoproliferation[203,204]. Disease presentation includes organ-specific autoimmunity and cytopenias, arthritis, enteropathy, non-malignant lymphoproliferation, and short stature due to GH deficiency[205]. Patients can present varying degrees of immunodeficiency, including infections with non-tubercular mycobacteria and fungi. The lymphoproliferation can mimic ALPS, including elevation of double-negative (TCR $\alpha\beta$ +CD4-CD8-) T cells. Cases mimicking CVID have also been reported[206]. Because STAT3 signals with JAK1/2 downstream of the IL-6 receptor, targeted therapy with JAKinibs and anti-IL6 monoclonal antibodies have been used successfully[207].

STAT1 variants have been associated with at least three different phenotypes: autosomal dominant gain-of-function (GOF) mutations cause CMC (IMD31C, OMIM #614162), autosomal dominant loss-of-function (LOF) variants cause isolated mycobacterial disease (IMD31A, OMIM

#614892), and autosomal recessive LOF mutations cause susceptibility to viral and mycobacterial infections (IMD31B, OMIM #613796). Moreover, STAT1 GOF CMC can present with a wide spectrum ranging from adult-onset isolated candidiasis, with or without CVID[208], to severe forms mimicking IPEX[209] or SCID[210].

SOCS1 haploinsufficiency was identified as a cause autosomal dominant predisposition to earlyonset autoimmunity characterized by cytokine hyper-responsiveness[211]. Interestingly, two children with the multisystem inflammatory syndrome (MIS-C) after SARS-CoV-2 infection were found to have SOCS1 deficiency[212]. The first patients identified in a large whole-genome sequencing cohort had a CVID diagnosis.[213]

PKCδ, encoded by **PRKCD**, is a kinase pivotal in the maintenance of B-cell tolerance. A homozygous mutation was first identified in a patient with hypogammaglobulinemia, anti-phospholipid autoantibodies syndrome, and glomerulonephritis[214]. The patient was classified as a CVID due to the lack of memory B cell and expanded CD21lo B cells. In the following years, other patients have been identified, all of whom presented as early-onset SLE, without features of immunodeficiency[215].

BACH2 is a transcription factor essential for T- and B-lymphocytes and was shown to be crucial in the inhibition of Tfh cells and control of autoimmunity[216,217]. Patients with autosomal dominant mutations leading to haploinsufficiency, develop BACH2-related immunodeficiency and autoimmunity (BRIDA). BRIDA presents with hypogammaglobulinemia, early-onset IBD, and diminished Tregs[218].

Between the disorders of immune dysregulation, there is also familial hemophagocytic lymphohistiocytosis (HLH) and a group of syndromes characterized by susceptibility to EBV-associated lymphoproliferation.

Familial HLH usually presents as a dramatic early-onset cytokine storm caused by an NK-cell cytotoxicity defect leading to uncontrolled positive feedback between activated macrophages and lymphocytes. The implicated gene mutations cause a deficiency in NK cells cytotoxic granules delivery, either by a lack of perforin (PRF1) or due to defective granule exocytosis (UNC13D, STXBP2, STX11). However, immunodeficiency or hypogammaglobulinemia are not features that are commonly reported in familial HLH. Nevertheless, several hypomorphic defects especially in **UNC13D** and **STXBP2** have been identified in patients with atypical late-onset HLH and hypogammaglobulinemia mimicking CVID[219].

X-linked lymphoproliferative disease (**XLP**), first described as "Duncan's disease" or "Purtilo syndrome", is caused by mutations of the **SH2D1A** gene on the X chromosome, encoding the SLAM-associated protein (SAP). SAP deficiency is characterized by the triad of fulminant mononucleosis with EBV-associated HLH, lymphoma, and hypogammaglobulinemia. Before exposure to EBV and in cases of hypomorphic variants, the clinical presentations of SH2D1A can be indistinguishable from CVID[220,221].

A subset of XLP families, without SH2D1A defects, was later found to harbor mutations in the Xlinked Inhibitor of Apoptosis (**XIAP**) gene[222]. The disease was called XLP2, and similarly to XLP1, it is characterized by a high rate of EBV-associated HLH. However, XLP2 patients don't show the predisposition to lymphoma that XLP1 patients have. XLP2 patients often develop early-onset IBD-like enteropathy[223]. A smaller proportion of XLP2 patients have also hypogammaglobulinemia, possibly mimicking CVID[224].

CD27/CD70 signaling pathway is a co-stimulatory regulator of T cells. Biallelic defects of both **CD27** and its ligand **CD70** are characterized by high susceptibility to EBV infection and lymphoproliferation, as well as varying degrees of immunodeficiency and hypogammaglobulinemia[225–228].

Autoinflammatory diseases (AID) are syndromes caused by spontaneous activation of the innate immune system, as opposed to autoimmunity defined as the inappropriate activation of the adaptive immune system. The spectrum of AID has considerably grown in the last decade to include pathologies caused by different mechanisms, such as cryopyrinopathies (IL-1), type I interferonopathies (IFNs), etc. In general, they manifest with recurrent fevers and inflammatory manifestations, such as vasculopathy and skin rashes. While immunodeficiency is not typical of AID, some do have hypogammaglobulinemia as an important feature and can mimic CVID in their presentation.

Phospholipase Cy2, encoded by the **PLCG2** gene, mediates signaling of B-cell, Fcy-receptors, mast cells, and NK cells. Monoallelic variants in PLCG2 have been associated to Phospholipase Cy2-Associated Antibody Deficiency and Immune Dysregulation (PLAID) and Auto-inflammation and phospholipase Cy2-Associated Antibody Deficiency and Immune Dysregulation (APLAID). Those mutations are temperature-sensitive gain-of-function mutations, leading to pathognomonic cold urticaria from evaporative cooling and associated with autoimmunity and humoral immune deficiency. Three patients from the original cohort fulfilled CVID criteria[229–232].

Adenosine deaminase 2, encoded by gene **ADA2** formerly known as CECR1, is an enzyme of purine metabolism. Unlike adenosine deaminase 1 (ADA1), which is the main enzyme converting deoxyadenosine to adenosine preventing toxic accumulation in lymphocytes leading to SCID, ADA2's physiologic role is less clear. ADA2 is expressed by activated monocytes, macrophages, and dendritic cells, and regulates inflammatory regulation. Deficiency of ADA2 (DADA2) was characterized as a pleiotropic autoinflammatory disease, causing early-onset strokes, polyarteritis nodosa-like vasculopathy, livedo reticularis, fevers, and hypogammaglobulinemia[233–235].

It became apparent early on that CVID was a very heterogeneous disease, and early classification efforts focused on the B cell phenotype[76]. The studies of the T cell phenotype in CVID led to the definition **of CID and LOCID** as separate entities. As discussed above, several criteria have been proposed to define CID/LOCID[10,79–81], the clinical and immunological features include T CD4+ lymphopenia, reduced naïve CD4+ T cells, proliferation deficits, or opportunistic infections. As such definitions are evolving and many patients that were first identified as CVID have been later re-classified as CID, many genetic defects that are in the CID category have been discovered in CVID cohorts. For example, ICOS deficiency, which was discovered as the first monogenic cause of CVID is classified today as CID.

Interleukin 21 (IL-21) is a pleiotropic cytokine of the common γ-chain family. It is produced by T and NKT cells, and its pivotal to Tfh function, and therefore GC formation. Biallelic mutations of both **IL21** and its receptor gene **IL21R** have been identified in patients with immunodeficiency[236,237]. The first identified patients had a diagnosis of CVID[238], but it was rapidly noted that they often suffer from opportunistic infections and severe immunodysregulation. Thus, IL-21 and IL-21R deficiency have been reclassified as CID[239,240].

The Caspase recruitment domain (CARD) / B-cell Lymphoma 10 (BCL10) / MALT1 paracaspase (MALT1) complex, also called the **CBM complex**, is a critical signaling hub downstream several receptors on immune and non-immune cells. Fine-tuning of the CBM complex is key to lymphocyte polarization and the function of innate immunity receptors. In recent years a wide spectrum of phenotypes, name CBMopathies, have been associated with mutations in the CBM proteins[241]. In lymphocytes the CBM complex, containing CARD11, signals downstream of the BCR and TCR, where it regulates IKK degradation and therefore canonical NFkB signaling.

At least three distinct phenotypes have been associated with variants of **CARD11**[242]. Biallelic loss-of-function (LOF) of CARD11 lead to a form of Severe Combined Immunodeficiency

(SCID)[243,244], mono-allelic dominant-negative LOF are associated with atopy and immunodeficiency (CADINS, CARD11-associated atopy with dominant interference of NF-κB signaling)[245], while gain-of-function (GOF) variants cause B-cell expansion with NF-κB and T-cell anergy (BENTA)[246,247]. Biallelic mutations in BCL10 and MALT1 were also associated with a few cases of CID, SCID, or IPEX[248–251].

BLK is an Src kinase involved in BCR-signaling. Mutations BLK have been found to segregate with CVID in one kindred. BLK deficiency perturbs B-cell proliferation and the ability to elicit antigen-specific CD4+ T[252].

VAV1 is a nucleotide guanine exchange factor that couples TCR activation to the actin cytoskeleton. A de novo heterozygous deletion of Vav1 was described in one patient with CID[253].

Surprisingly, with the use of unbiased genetic approaches, mutations in genes that were traditionally associated with **Severe Combined Immunodeficiency (SCID)** were found in patients with relatively mild defects, often fulfilling CVID criteria. Those mutations are generally compound heterozygous hypomorphic variants, effects of somatic reversion, or X-linked disorders in females with skewed lyonization.

The prototypical example is compound heterozygous mutations of the Recombinase genes **RAG1** and **RAG2**, which have been widely studied as the cause of T-B-NK+ SCID. RAG genes are fundamental for VDJ recombination, and therefore the maturation of T and B cells. Experiments showed that some missense variants can retain varying degrees of recombinase activity. The residual functionality was shown to correlate with the severity of the phenotype. Patients with null variants presented with the classical SCID, low residual activity was associated with Omenn syndrome, and patients with hypomorphic defects were found to exhibit a characteristic phenotype, named CID with granulomatous disease and autoimmunity (CID-G/AI)[254]. Cases of CID-G/AI have been reported with onset in adulthood, with clinical phenotypes ranging from CVID to selective IgA deficiency[255,256].

Artemis, encoded by the **DCLRE1C** gene, is involved in non-homologous end-joining and is essential to VDJ recombination in T and B cells. Biallelic mutations of Artemis cause T-B-NK+ SCID with increased radiosensitivity. Similar to RAG, hypomorphic variants have been found in patients with predominant humoral defects clinically mimicking CVID.[257]

JAK3 signaling is crucial for differentiation and survival of hematopoietic lineage cells, as it signals downstream of the common gamma chain, shared by cytokine receptors such as IL-2, IL-

4, IL-7, IL-9, IL-15, and IL-21. Biallelic defects of **JAK3** cause autosomal recessive T-B+NK- SCID. Few patients with hypomorphic mutations, retaining partial signaling activity, were described as mimicking CVID.[258]

A humoral defect, mimicking CVID, can be the presenting feature of **cancer predisposition syndromes and bone marrow failure syndromes.** Some examples were already discussed above, such as hematological malignancies in IKZF1 haploinsufficiency, EBV-lymphoproliferative disease (XLP1, CD27, etc.), CSR defects associated with DNA repair defects (MSH, PMS2, etc.).

GATA2 is a zinc finger transcription factor essential for hematopoiesis and lymphatic angiogenesis. **GATA2** deficiency and haploinsufficiency is a pleiotropic syndrome, causing a wide spectrum of phenotypes including viral and bacterial infections, cytopenias, myelodysplasia, myeloid leukemias, pulmonary alveolar proteinosis, and lymphedema. The age of onset ranges from infancy to late adulthood. Immunologically they present monocytopenia, reduction of B lymphocytes and NK cell in particular the CD56bright subset[259]. GATA2 deficiency is also known as MonoMAC syndrome, for monocytopenia and non-tubercular mycobacteria[260], and Emberger syndrome, primary lymphedema associated with a predisposition to acute myeloid leukemia[261]. One of the features is B cell deficiency and hypogammaglobulinemia. Because of the invariable progression of the disease to myeloid malignancies, treatment with hematopoietic stem cell transplantation is highly recommended, and therefore early recognition is vital[262,263].

Fanconi anemia (FA) is caused by defects in a group of proteins responsible for DNA repair via homologous recombination. It is the prototypical bone marrow failure syndrome, characterized by aplastic anemia, cancer predisposition, and congenital defects. Two patients with biallelic FANCA and FANCE mutations with a diagnosis of adult-onset CVID have been described, they had no anemia, neutropenia, or thrombocytopenia. **FANC** mutations can cause isolated immunodeficiency in addition to bone marrow failure and malignancy[264]. Interestingly, also BRCA genes, better known for their autosomal dominant breast-cancer risk, are part of the FA gene group, and mutations have been associated with FA and immunodeficiency[265].

2. Objectives

The goal of this project is to recruit a cohort of CVID patients and characterize them clinically, immunologically, and genetically. For this purpose, the project can be divided into the following steps:

- 1) Recruitment of the patients through detailed clinical assessment and in-depth immunological screening with flow cytometry.
- 2) WES of the selected patients (and their relatives), filtering first to known PID genes, followed by an analysis based on genetic and phenotypic prioritizing bioinformatics tools.
- 3) Functional validation of selected variants of unknown significance.

The project is geared towards

- CVID patients and their families with complex phenotypes and in whom a genetic diagnosis could improve quality of life and give relevant information regarding the heritability of their disease.
- Aid clinical immunologists in the management of those complex patients, offering prognostic information, risk stratification and opportunities for targeted therapies
- Researchers in the fields of immunology, discovering novel mechanisms of immune deficiency and dysregulation and offering unique *in vivo* models

3. Methods

3.1. Recruitment

Subjects diagnosed with CVID or CID using the referral center established criteria, and in accordance with the European Society of Immuno Deficiencies (ESID) registry criteria for CVID and CID respectively, were recruited.

CVID ESID registry criteria

At least one of the following:

- increased susceptibility to infection
- autoimmune manifestations
- granulomatous disease
- unexplained polyclonal lymphoproliferation
- affected family member with antibody deficiency

AND marked decrease of IgG and marked decrease of IgA with or without low IgM levels (measured at least twice; of IgA with or without low IgM levels (measured at least twice; <2SD of the normal levels for their age);

AND at least one of the following:

- poor antibody response to vaccines (and/or absent isohemagglutinins); i.e., absence
 of protective levels despite vaccination where defined
- low switched memory B cells (<70% of age-related normal value)

AND secondary causes of hypogammaglobulinemia have been excluded (e.g., infection, protein loss, medication, malignancy)
AND diagnosis is established after the 4th year of life (but symptoms may be present before) **AND** no evidence of profound T-cell deficiency, defined as 2 out of the following (y=years of life):

- CD4 numbers/microliter: 2-6y <300, 6-12y <250, >12y <200
- % naive of CD4: 2-6y <25%, 6-16y <20%, >16y <10%
- T cell proliferation absent

CVID ESID registry criteria

At least one of:

- at least one severe infection (requiring hospitalization)
- one manifestation of immune dysregulation (autoimmunity, IBD, severe eczema lymphoproliferation, granuloma)
- malignancy
- affected family member

AND 2 of 4 T cell criteria fulfilled:

- reduced CD3 or CD4 or CD8 T cells (using age-related reference values)
- reduced naïve CD4 and/or CD8 T cells
- elevated g/d T cells
- reduced proliferation to mitogen or TCR stimulation

AND HIV excluded

AND exclusion of a clinical diagnosis associated with CID (e.g., defined syndromic diseases, DKC, AT, CHH)

Patients were enrolled after the internal ethical board review of the Careggi University Hospital approved the protocol of this study. When available, samples from parents and siblings of CVID patients were submitted for whole-exome to study familial segregation. The recruitment centers were the following:

- SOD Immunology and Cellular Therapies and SOD Immunoallergology, AOU Careggi, Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy.
- Center for Rare Immunological and Cardiovascular Diseases, Behçet Center and Lupus Clinic, AOU Careggi, Florence, Italy.
- Clinical Immunology and Allergy Unit, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy.

Genetic testing was performed at:

• Medical Genetics Unit, Anna Meyer Children's University Hospital, Florence, Italy.

Phenotypic and functional immunologic testing was carried by:

• Center of Flow Cytometry and Immunotherapy (CDCI), AOU Careggi, Florence, Italy.

Family history was used to build pedigrees for every proband. Pedigree data were visualized using the kinship2 R package[266].

3.2. Flow cytometry

All analyses were performed from fresh blood samples drawn in the previous 24 hours and conserved at room temperature, which was shown to yield comparable results to gradient isolated peripheral blood mononuclear cells[267].

Four eight-color panels were used to screen circulating lymphocyte populations and subpopulations (Table 1).

Panel 1 (TBNK) is a standard lymphocyte population panel, including CD45, CD3, CD4, CD8, HLA-DR, CD19, CD16, and CD56, geared to analyze T cells and their activation (HLA-DR), B cells, NK and NKT cells. The gating strategy is exemplified in Fig. 1.



Figure 1, Gating Strategy for the first panel of a healthy donor.

Panel 2 (B) analyzes B cell subpopulations using CD45, CD19, CD20, IgM, IgD, CD38, CD27 and CD21. It enables the differentiation of B naïve, switched memory, marginal zone-like, plasmablasts, transitional, and CD21low B cells. Paris, Freiburg, and EUROclass classification



criteria were calculated as described in Piqueras et al[77], Warnatz et al.[76] and Wehr et al.[78], respectively. The gating strategy is shown in Fig. 2.

Figure 2, Gating Strategy for the second panel of a healthy donor.

Panel 3 (T1) includes CD45, CD3, CD4, CD8, TCR $\alpha\beta$, TCR $\gamma\delta$, CD25 and CD127. It enables to identify TCR $\alpha\beta$, TCR $\gamma\delta$ T cells, DNT T cells, and Treg cells. The gating strategy is represented in Fig. 3.



Figure 3, Gating Strategy for the third panel of a healthy donor.

Panel 4 (T2) studies T cell differentiation by using CD45, CD3, CD4, CD8, CD45RA, CCR7, CD31, and CD57. It recognizes T CD4 and CD8 subpopulation based on the expression of CCR7, CD45RA (CCR7+CD45RA naïve, CCR7+CD45RA- central memory, CCR7-CD45RA- effector memory, CCR7-CD45RA+ terminal effector memory), CD31 (recent thymic emigrants), and CD57 (senescent). The gating strategy is shown in Fig 4.



Figure 4, Gating Strategy for the fourth panel of a healthy donor.

Panel 5 (Tfh) analyzes T follicular helper cells and their phenotype by using CD45, CD3, CD4, CD45RA, CXCR5, CCR6, CXCR3, and CCR7. Tfh cells are CD3+CD4+CD45RA-CXCR5+, Tfh subpopulations are based on the expression of CXCR3 and CCR6, Tfh1 (CCR6-CXCR3+), Tfh2 (CCR6-CXCR3-), Tfh17/1 (CCR6+CXCR3+). The gating strategy is shown in Fig. 5.



Figure 5, Gating Strategy for the fifth panel of a healthy donor.

Eight-color tubes were acquired on FACS Canto II (BD Biosciences). Data were analyzed with FACS Diva (BD Biosciences) and FlowJo Software (BD Biosciences).

Antibodies	Fluorochromes	Producer	Clone
lgM	FITC	BD PHARMINGEN	Mouse anti-IgM
lgD	PE	BD PHARMINGEN	Mouse anti-IgD
CD19	PERCP	BD	SJ25C1
CD38	PECY7	BD	HB7
CD27	APC	BD	L128
CD20	APCH7	BD	L27
CD21	V450	BD	BLY4
CD45	V500	BD	2D1
ΤCRαβ	FITC	BD	WT31
ΤCRγδ	PE	BD	11F2
CD3	PERCP	BD	SK7
CD25	PECY7	BD	2A3
CD8	APC	BD	SK1
CD4	APCH7	BD	SK3
CD127	V450	BD HORIZON	HIL7RM21
CD57	FITC	BD	HNK1
CD8	PE	BD	SK1
CD45RA	PECY7	BD	L48
CD31	APC	BD PHARMINGEN	WM56

CCR7	V450	BD HORIZON	150503
CXCR3	FITC	R&D SYSTEM	49801
CXCR5	PE	R&D SYSTEM	51505
CCR6	APC	BD PHARMINGEN	11AG

Table 1, Antibodies used in the flow cytometry panels.

3.3. Statistical analysis

Statistical analysis was done using R (R version 4.0.2) and RStudio (Version 1.3.1073), using Tidyverse packages and Rstatix. To compare the frequency of categorical variables Fisher's exact test was used with Holm correction. For comparisons across multiple groups, Kruskal-Wallis test was used. Posthoc pairwise analysis was done with Dunn's test with Benjamini-Hochberg correction. To compare between two groups Mann-Whitney-Wilcoxon rank-sum Test and Benjamini-Hochberg correction were used.

3.4. Whole-exome sequencing

DNA extraction: Peripheral blood DNA was extracted using QIAamp Mini Kit (QIAGEN[®], Hilden, Germany) according to manufacturer's instructions and quantified by NanoDROP 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

DNA library preparation: For Exome Sequencing, a strategy based on enzymatic fragmentation to produce dsDNA fragments followed by End repair, A-tailing, adapter ligation, and library amplification was used. Libraries were hybridized with the protocol SeqCap EZ Exome v3 and sequenced with the platform HiSeq 1000.

Assembly, Variant Calling, and Identification of pathogenic variants: Reads were aligned with the human reference hg19 genome using Burrows-Wheeler Aligner (BWA). Coverage statistics were obtained with an in-house developed script. The reads produced were mapped to the reference genome and were analyzed with the IGV (Integrative Genome Viewer, 2013 Broad Institute) software. At the same time, the variant call for identification of nucleotide variants was performed using the Genome Analysis ToolKit (GATK) Unified Genotyper Module. The average target depth was 100x *per* sample.

Variant Annotation: VCF annotations were performed using ANNOVAR and VarAFT, all variants were annotated with dbSNP v150, GnomAD, KAVIAR, 1000 genomes, ClinVar and COSMIC databases. Prediction scores calculated included CADD, DANN, EIGEN, FATHMM, GERP++, LRT, MetaIR, MetaSVM, MetationTaster, MutationAssessor, Polyphen2, PROVEAN, SIFT, SiPhy, UMD, and VEST3.

In-silico CNV Prediction: CNV Calling was performed using ExomeDepth. For every sample, a reference group was created excluding samples from relatives. CNV variants in the X chromosome were called separately using sex-restricted reference groups. Raw CNV calls were

exported as .bed files to ANNOTSV for annotation. CNVs were ordered by the Bayes Factor (BF) generated by ExomeDepth, then known/benign CNV annotated from ANNOTSV were filtered out. CNVs encompassing genes from the candidate gene list were prioritized.

Filtering and Prioritization strategies: Patient exomes were filtered for mutations in 541 genes, containing the genes from the IUIS 2019 IEI classification[4], the "Primary Immunodeficiency" (Version 2.384) PanelApp gene panel, and candidate genes from experimental evidence (Supplementary table). Heterozygous and homozygous mutations were excluded if the allele frequencies in the general population were >1.0% in the GnomAD Exome database. Familial segregation was studied when samples were available. Candidate mutations were manually confirmed by examining read alignment in the integrated genomics viewer (IGV; Broad Institute).

Variant Classification: The initial classification of identified variants was performed according to the five-tier scheme as recommended by The American College of Medical Genetics and Genomics[124].

4. Results

4.1 Patients

A cohort of 44 CVID patients was recruited and 23 first-degree relatives, between those 11 affected by CVID or an associated condition (e.g. autoimmune cytopenias, IgA deficiency) and 12 unaffected. 12 kindreds (27%) had a familial history of immunodeficiency, 32 were sporadic cases (73%). Between the probands, there was an even distribution of males and females (22 males, 22 females), the average age is 43.2±13 (min 22, max 75, median 42, IQR 16.5).

Patients were divided into "Infection-only" or "complex" according to the clinical manifestations. Patients with any autoimmune, lymphoproliferative, or neoplastic disease were considered complex. 33 Probands were classified as complex (75%), and 11 as infection-only (25%).

	n	%		n	%
Respiratory infections	35	0.80	Autoimmunity	25	0.57
Chronic Rhinosinusitis	15	0.34	Interstitial Lung disease	1	0.02
Otitis media	9	0.20	Enteropathy	15	0.34
Bronchitis	24	0.55	Hepatitis	3	0.07
Pneumonia	22	0.50	Cholangitis	2	0.05
Bronchiectasis	18	0.41	Autoimmune cytopenias	8	0.18
Chronic Diarrhea	18	0.41	Thrombocytopenia	9	0.20
Encephalitis	1	0.02	Hemolytic anemia	2	0.05
Chronic Candidiasis	2	0.05	Neutropenia	3	0.07

Verrucosis	1	0.02	Vitiligo	1	0.02
			Psoriasis	3	0.07
Lymphoproliferation	28	0.64	Alopecia	1	0.02
Splenomegaly	25	0.57	Atrophic gastritis	2	0.05
Splenectomy	5	0.11	Autoimmune Thyroiditis	2	0.05
Hepatomegaly	9	0.20			
Portal hypertension	4	0.09	Hematologic neoplasia	5	0.11
Chronic Lymphadenopathies	16	0.36	Solid neoplasia	2	0.05
Allergy	7	0.16			
Atopic dermatitis	4	0.09			
Asthma	2	0.05			
Food allergy	1	0.02			
Inhalants allergy	2	0.05			

Table 2, Clinical features of the probands, number and percentage of the cohort presenting with a history of infections, lymphoproliferation, allergy, autoimmunity, and neoplasia.

4.2. Immunological phenotype

We evaluated the differences between lymphocyte populations between the probands and the healthy relatives (controls), affected relatives were excluded. Patients had a significantly higher proportion of T CD8+ cells and lower NK cells. Between NK cells patients had a significantly higher percentage of CD56bright cells. T CD4+ cells were lower, but significance was not met after adjusting for multiple comparisons.

B cells were comparable between patients and controls, but -as expected- patients had markedly reduced switched memory B cells and plasmablasts, reflecting the humoral deficiency.

Differences in the maturation subpopulations of T CD4+ and T CD8+ did not reach statistical significance due to the large heterogeneity of the patients. However, a subgroup of patients has markedly reduced T CD4+ naïve lymphocytes. T follicular helper cells were significantly higher in patients compared to controls, however, there wasn't a significant difference between Tfh subpopulations.

Subpopulations	Patients	(n=41)	Controls (n=7)					
	mean	sd	mean	sd	р	p.signif	p.adj	p.adj.signif
Lymphocytes	0.28	0.119	0.296	0.089	4.4e-01	ns	0.6000	ns
T cells	0.83	0.106	0.763	0.067	5.4e-02	ns	0.2206	ns
T CD4	0.417	0.128	0.512	0.043	1.7e-02	*	0.0887	ns
T CD4 HLA-DR+	0.114	0.109	0.053	0.022	4.1e-02	*	0.1863	ns
T CD8	0.359	0.159	0.213	0.052	5.3e-03	**	0.0412	*
T CD8 HLA-DR+	0.301	0.205	0.175	0.068	1.1e-01	ns	0.2870	ns
B cells	0.088	0.069	0.098	0.045	4.4e-01	ns	0.6000	ns
NK cells	0.076	0.071	0.134	0.058	5.2e-03	**	0.0412	*
NK CD56 ^{bright}	0.089	0.066	0.023	0.011	1.4e-03	**	0.0198	*

NK CD56 ^{dim}	0.733	0.173	0.881	0.062	1.1e-02	*	0.0644	ns
NKT cells	0.098	0.075	0.073	0.028	8.4e-01	ns	0.8841	ns
B Naive	0.808	0.164	0.682	0.195	9.3e-02	ns	0.2870	ns
MZL	0.122	0.149	0.121	0.121	4.0e-01	ns	0.6000	ns
Switched Memory	0.027	0.032	0.156	0.076	7.8e-05	****	0.0032	**
CD21 ^{lo}	0.198	0.197	0.087	0.032	2.9e-01	ns	0.4731	ns
Transitional	0.137	0.195	0.033	0.02	3.0e-01	ns	0.4731	ns
Plasmablasts	0	0.002	0.001	0.001	1.2e-03	**	0.0198	*
CD4 Naive	0.27	0.179	0.366	0.113	1.7e-01	ns	0.3030	ns
CD4 TCM	0.318	0.09	0.297	0.06	4.7e-01	ns	0.6269	ns
CD4 TEM	0.387	0.188	0.314	0.101	4.3e-01	ns	0.6000	ns
CD4 TEMRA	0.025	0.022	0.023	0.014	1.0e+00	ns	1.0000	ns
CD4 RTE	0.232	0.14	0.268	0.124	5.7e-01	ns	0.7069	ns
Treg	0.082	0.058	0.056	0.01	1.6e-01	ns	0.3030	ns
CD8 Naive	0.219	0.176	0.229	0.162	6.8e-01	ns	0.7679	ns
CD8 TCM	0.052	0.038	0.076	0.048	1.4e-01	ns	0.2870	ns
CD8 TEM	0.382	0.176	0.353	0.138	6.9e-01	ns	0.7679	ns
CD8 TEMRA	0.347	0.17	0.342	0.163	9.2e-01	ns	0.9420	ns
CD8 CD57+	0.361	0.205	0.221	0.197	7.5e-02	ns	0.2788	ns
TCRαβ	0.937	0.053	0.962	0.015	1.4e-01	ns	0.2870	ns
TCRαβ DNT	0.021	0.026	0.013	0.011	1.7e-01	ns	0.3030	ns
TCRγδ	0.06	0.053	0.035	0.014	1.3e-01	ns	0.2870	ns
T conv	0.525	0.142	0.495	0.084	7.3e-01	ns	0.7833	ns
T conv1	0.106	0.117	0.066	0.1	1.4e-01	ns	0.2870	ns
T conv2	0.428	0.131	0.452	0.173	6.7e-01	ns	0.7679	ns
T conv17	0.369	0.139	0.4	0.121	6.6e-01	ns	0.7679	ns
T conv17/1	0.097	0.069	0.081	0.11	1.4e-01	ns	0.2870	ns
Tfh	0.171	0.095	0.078	0.044	6.0e-03	**	0.0412	*
Tfh1	0.182	0.14	0.121	0.128	1.1e-01	ns	0.2870	ns
Tfh2	0.337	0.103	0.364	0.128	5.5e-01	ns	0.7034	ns
Tfh17	0.384	0.151	0.459	0.118	3.0e-01	ns	0.4731	ns
Tfh17/1	0.097	0.06	0.057	0.054	1.2e-01	ns	0.2870	ns

Table 3, Relative frequencies of lymphocyte populations in the probands compared to the controls. Significance levels: $p \ge 0.05$ ns; p < 0.01 **; p < 0.001 ***; p < 0.001 ****.

We then investigated the differences between patients with an infection-only and a complex phenotype. There was no statistically significant difference after adjustment. The unadjusted data is compatible with the literature, finding a reduced B memory compartment (switched and marginal zone-like) and a reduction in T CD4+ naïve cells in complex patients.

Subpopulations	Infections-	only (n=11)	Comple	x (n=30)				
	mean	sd	mean	sd	р	p.signif	p.adj	p.adj.signif
Lymphocytes	0.282	0.094	0.279	0.128	0.517	ns	0.66	ns
T cells	0.799	0.098	0.841	0.109	0.211	ns	0.38	ns
T CD4	0.449	0.086	0.406	0.14	0.245	ns	0.40	ns
T CD4 HLA-DR+	0.07	0.033	0.13	0.122	0.137	ns	0.32	ns

T CD8	0.294	0.095	0.383	0.172	0.112	ns	0.30	ns
T CD8 HLA-DR+	0.217	0.126	0.331	0.221	0.154	ns	0.32	ns
B cells	0.107	0.025	0.081	0.078	0.099	ns	0.29	ns
NK cells	0.089	0.081	0.071	0.068	0.653	ns	0.74	ns
NK CD56 ^{bright}	0.08	0.056	0.092	0.071	0.860	ns	0.89	ns
NK CD56 ^{dim}	0.727	0.165	0.735	0.179	0.895	ns	0.90	ns
NKT cells	0.085	0.048	0.103	0.083	0.873	ns	0.89	ns
B Naive	0.757	0.118	0.826	0.176	0.064	ns	0.22	ns
MZL	0.158	0.11	0.109	0.16	0.041	*	0.22	ns
Switched Memory	0.051	0.05	0.019	0.018	0.013	*	0.22	ns
CD21 ^{lo}	0.101	0.069	0.234	0.217	0.119	ns	0.30	ns
Transitional	0.052	0.05	0.169	0.218	0.287	ns	0.44	ns
Plasmablasts	0	0	0	0.002	0.024	*	0.22	ns
CD4 Naive	0.333	0.146	0.248	0.187	0.158	ns	0.32	ns
CD4 TCM	0.361	0.095	0.302	0.085	0.060	ns	0.22	ns
CD4 TEM	0.287	0.103	0.423	0.2	0.029	*	0.22	ns
CD4 TEMRA	0.019	0.011	0.027	0.024	0.471	ns	0.64	ns
CD4 RTE	0.294	0.139	0.21	0.136	0.077	ns	0.24	ns
Treg	0.094	0.061	0.077	0.057	0.181	ns	0.35	ns
CD8 Naive	0.304	0.174	0.188	0.168	0.054	ns	0.22	ns
CD8 TCM	0.059	0.03	0.049	0.04	0.139	ns	0.32	ns
CD8 TEM	0.362	0.137	0.389	0.19	0.746	ns	0.83	ns
CD8 TEMRA	0.275	0.115	0.374	0.18	0.064	ns	0.22	ns
CD8 CD57+	0.286	0.166	0.389	0.213	0.195	ns	0.36	ns
TCRαβ	0.937	0.027	0.937	0.06	0.418	ns	0.59	ns
TCRαβ DNT	0.016	0.012	0.022	0.029	0.627	ns	0.73	ns
TCRγδ	0.058	0.029	0.06	0.06	0.627	ns	0.73	ns
T conv	0.436	0.094	0.557	0.144	0.042	*	0.22	ns
T conv1	0.124	0.128	0.099	0.115	0.571	ns	0.71	ns
T conv2	0.352	0.111	0.456	0.128	0.034	*	0.22	ns
T conv17	0.383	0.151	0.364	0.136	0.517	ns	0.66	ns
T conv17/1	0.141	0.092	0.081	0.051	0.046	*	0.22	ns
Tfh	0.193	0.086	0.163	0.098	0.517	ns	0.66	ns
Tfh1	0.184	0.161	0.181	0.135	0.211	ns	0.38	ns
Tfh2	0.282	0.083	0.357	0.103	0.245	ns	0.40	ns
Tfh17	0.416	0.171	0.373	0.145	0.137	ns	0.32	ns
Tfh17/1	0.118	0.07	0.089	0.055	0.112	ns	0.30	ns

Table 4, Relative frequencies of lymphocyte populations in the probands divided into infection-only or complex phenotype. Significance levels: p=> 0.05 ns; p<0.05 *; p<0.01 **; p<0.001 ***; p<0.0001 ****.

Probands were subdivided according to the Freiburg[76], Paris[77], and EUROClass[78] classifications for B cell phenotype and the Giovannetti[79] classification for T naïve cells. We compared the frequency of "infection-only" and "complex" patients in each category.



Figure 6, Clustering of scaled relative lymphocyte populations across the probands, divided into "infection-only" and "complex" phenotype, and controls. On the right pane assignment to the Giovannetti, Paris, Freiburg and EUROClass classification schemes.

Patients with infections-only were significantly associated with Freiburg class II, while patients with a complex phenotype were significantly associated with the Paris MB0 group. We did not find a significant association with the EUROClass and Giovannetti classifications.

Freiburg	Total	complex	infection-only	р	p.adj p.adj.signif
la	9.3% (4)	9.3% (4)	0.0% (0)	0.5580	0.558 ns
Ib	25.6% (11)	25.6% (11)	0.0% (0)	0.0408	0.082 ns
П	65.1% (28)	39.5% (17)	25.6% (11)	0.0077	0.023 *
Paris	Total	complex	infection-only	p	p.adj p.adj.signif
MBO	55.8% (24)	51.2% (22)	4.7% (2)	0.0053	0.016 *
MB1	39.5% (17)	23.3% (10)	16.3% (7)	0.0800	0.122 ns
MB2	4.7% (2)	0.0% (0)	4.7% (2)	0.0609	0.122 ns
Giovannet	ti Total	complex	infection-only	р	p.adj p.adj.signif
Group I	29.3% (12) 26.8% (1	1) 2.4% (1)	0.13	0.38 ns
Group II	26.8% (11) 17.1% (7	7) 9.8% (4)	0.44	0.89 ns

Group III	43.9% (18)	29.3% (12)	14.6%	6 (6)	0.49	0.89 ns
EUROClass	-	Total	complex	infection-only	р	p.adj p.adj.signif
В-	-	16.3% (7)	16.3% (7)	0.0% (0)	0.163	0.98 ns
B+ SmB- Trhigh (CD21lo	2.3% (1)	2.3% (1)	0.0% (0)	1.000	1.00 ns
B+ SmB- Trhigh (CD21norm	14.0% (6)	11.6% (5)	2.3% (1)	1.000	1.00 ns
B+ SmB- Trnorm	CD21lo	18.6% (8)	14.0% (6)	4.7% (2)	1.000	1.00 ns
B+ SmB- Trnorm	CD21norm	4.7% (2)	4.7% (2)	0.0% (0)	1.000	1.00 ns
B+ SmB+ CD21lo		27.9% (12)	18.6% (8)	9.3% (4)	0.467	1.00 ns
B+ SmB+ CD21nc	orm	16.3% (7)	7.0% (3)	9.3% (4)	0.058	0.41 ns

Table 5, Distribution of Total probands, and divided onto "infection-only" and "complex", across the Giovannetti, Paris, Freiburg and EUROClass classification schemes. Significance levels: p<0.05 *; p<0.01 **; p<0.001 ***; p<0.0001 ****.



Figure 7, Distribution of relative lymphocyte subpopulation frequencies across patients and controls. Significance levels: p<0.05 *; p<0.01 **; p<0.001 ***; p<0.0001 ****.



Figure 8, Distribution of relative lymphocyte subpopulation frequencies across patients and controls. Significance levels: p<0.05 *; p<0.01 **; p<0.001 ***; p<0.0001 ****.

4.3. WES results

After sequence alignment and variant calling, 38 proband patient exomes and their available relatives, were filtered to identify variants in the list of candidate immunodeficiency-associated genes. The variants in the gene panel were then filtered for rare (<1% from GnomAD) exonic or splicing, non-synonymous variants identifying 732 candidate variants (mean 18.3±4.1/patient).

The variants were then classified and manually filtered to identify Pathogenic, Likely Pathogenic, and VUS coherent with disease inheritance (biallelic, monoallelic) and family history. A putative pathogenic variant was therefore identified in 50% (16/38) of the probands.

We identified 14 Variants in 8 genes (CARD11, CD40LG, CTLA4, NFKB2, NFKB1, PIK3CD, PTEN, and TCF3), across 13 patients, 12 monoallelic variants, and 1 biallelic variant. In 7 patients we found 8 variants of TNFRSF13B (TACI), the mutations were biallelic in two patients (1 homozygous, 1 compound heterozygous). In 2 patients with other mutations (PIK3CD and PTEN), we also identified the p.H159Y TNFSF13C (BAFFR) variant.



Figure 9, Percentage of probands in whom at least one candidate genetic variant was identified, divided into single genes and their overall frequency in the cohort.

id	ref_gene	ref_trans	exon	coding_seq	prot_seq	Genotype	dbSNP	GnomAD_AF	CADD	- ClinVar	ACMG Classification
014A	CARD11	NM_032415	exon18	c.2449G>A	p.A817T	het	rs376539147	5.572e-05	9.682	Uncertain_significance	VUS
019A			exon21	c.2759G>T	p.R920L	het		0	24.4		VUS
			exon16	c.2066G>A	p.G689E	het			27.3		VUS
031A			exon16	c.1975G>A	p.V659M	het	rs78443994	0.0002	10.96	Uncertain_significance	VUS
034A	CD40LG	NM_000074	exon1	c.107T>A	p.M36K	hom	rs104894774		23.7	Likely_pathogenic	Pathogenic
009A	CTLA4	NM_005214	exon3	c.567G>A	p.M189I	het	•	•	18.24		Likely Pathogenic
025A			exon2	c.164G>A	p.S55N	het			7.553		VUS
036A			exon2	c.440C>A	p.T147N	het			25.9		Likely Pathogenic
032A	NFKB1	NM_001165412	exon10	c.897dupT	p.D299fs	het	rs773694113	3.983e-06		Pathogenic/Likely_pathogenic	Pathogenic
032B			exon10	c.897dupT	p.D299fs	het	rs773694113	3.983e-06		Pathogenic/Likely_pathogenic	Pathogenic
021A	NFKB2	NM_001261403	exon13	c.1340A>G	p.N447S	het		4.155e-05	12.49	Uncertain_significance	VUS
005A	PIK3CD	NM_001350235	exon12	c.1555C>T	p.R519W	het	rs780269932	2.953e-05	26.7		VUS
030A	PTEN	NM 000314	exon6	c.596T>C	p.M199T	het	rs587781538		10.53	Uncertain significance	VUS
		_									
024A	TCF3	NM_001136139	exon11	c.928C>T	p.P310S	het	rs531068508	4.882e-06	23.5		VUS

Table 6, Selected rare candidate Pathogenic, Likely Pathogenic or Variants of Uncertain Significance (VUS) identified in genes from the PID-associated gene panel.

id	ref_gene	ref_trans	exon	coding_seq	prot_seq	genotype	dbSNP	- GnomAD_AF	CADD
004A	TNFRSF13B	NM_012452	exon4	c.512T>G	p.L171R	het	rs143027621	0.0001	25.4
016A			exon3	c.310T>C	p.C104R	het	rs34557412	0.0035	25.9
022A			exon3	c.204dupA	p.L69fs	het	rs72553875	0.0004	
			exon4	c.554T>G	p.F185C	het	rs778227535	7.974e-06	25.0
033A			exon3	c.310T>C	p.C104R	hom	rs34557412	0.0035	25.9
038A			exon3	c.310T>C	p.C104R	het	rs34557412	0.0035	25.9
040A			exon3	c.260T>A	p.187N	het	rs72553877	0.0005	25.2
042A			exon3	c.310T>C	p.C104R	het	rs34557412	0.0035	25.9
005A	TNFRSF13C	NM_052945	exon3	c.475C>T	p.H159Y	het	rs61756766	0.0057	27.6
030A			exon3	c.475C>T	p.H159Y	het	rs61756766	0.0057	27.6

Table 7, Rare variants identified in the disease-associated TNFRSF13B (TACI) and TNFRSF13C (BAFFR) genes.

4.3.1. In-silico CNV Prediction

CNV predicted In-silico with ExomeDepth and annotated with ANNOTSV were filtered to identify variants identified with high-confidence and/or encompassing candidate genes. 4 CNV, 3 deletions encompassing candidate genes (TBX1 and CTLA), and one large duplication in the 19p13 region were selected for further analysis.

Two patients were predicted to have microdeletions in the TBX1 gene, in the 22q11.2 region. Because of the small size (964bp and 243bp), these deletions will need to be confirmed with a microarray. Neither of the patients had a reported history of hypoparathyroidism, dysmorphism, or cardiac anomalies. However, one of them, a 44 years old female has a CID phenotype (markedly reduced naïve T CD4+) and suffers from severe GLILD, both features compatible with a 22q11 deletion syndrome[59,268,269].

A deletion of 225Kb in the 2q33 region, encompassing the CD28, CTLA, and ICOS genes, was predicted with high confidence in one patient. Interestingly, the three genes all belong to the CD28-superfamily, however, monoallelic defects of CD28 and ICOS are not pathogenic. Haploinsufficiency of CTLA4 is a known disease mechanism. Moreover, several patients with deletions in the cluster have been reported[195,270].

One patient was predicted to have a 378Kb duplication in the 19p13 region. Patients with larger duplications (~7Mb) in this locus have been reported with Facial dysmorphia, Urogenital malformation, growth, and neurodevelopmental Retardation, Immunodeficiency, trisomy 19p13 (FURID19)[271]. However, ExomeDepth specificity for duplication is lower than for deletions, therefore the CNV needs to be confirmed with an array CGH.

With exception of the CTLA4 deletion, the CNVs have not been counted towards the number of patients with identified pathogenic variants as they need to be further confirmed by Sanger sequencing or array CGH.

id	Chr	Start	End	Size	CNV	Genes
003A	chr22	19753426	19754390	964bp	DEL	TBX1
027A	chr22	19753282	19753525	243bp	DEL	TBX1
022A	chr2	204599508	204824322	225Kb	DEL	CD28/CTLA4/ICOS
007A	chr19	6213054	6591013	378Kb	DUP	ACER1/ALKBH7/CD70/CLPP/CRB3/DENND1C/ GTF2F1/KHSRP/LOC390877/MIR3940/MIR6790/ MIR6885/MLLT1/PSPN/SLC25A23/SLC25A41/ TNFSF9/TUBB4A

Table 8, Deleterious CNV predicted by ExomeDepth in the probands.

4.3.2. CARD11

We identified 3 patients with variants in CARD11, 2 monoallelic, and one heterozygous compound.

The p.V659M variant replaces a valine with methionine at codon 659 of the CARD11 protein. The valine residue is moderately conserved, and the substitution is not predicted to be deleterious. Moreover, this variant allele frequency is relatively high (GnomAD 0.2%). It was already reported in an individual affected with sinopulmonary/skin infections and pneumonia reported by Dorjbal et al.[245]. However, In the same study, the variant was found to not affect protein function[245].

This p.A817T variant replaces an alanine with threonine at codon 817. The alanine residue is moderately conserved, and the substation is predicted to be tolerated by most bioinformatics tools. This variant is present in population databases (GnomAD 0.0056%) and was not reported in patients with diseases associated with CARD11. No functional data is available.

In summary, the available evidence is currently insufficient to determine the role of these variants in the disease, and they are both classified as VUS.

Patient	Variants	Age	Sex	Infections	Other conditions	lgG/A/M	EUROClass	T naive
019A	CARD11 p.R920L p.G689E	42	F	Respiratory, Zoster oticus, Herpes simplex	Atopic dermatitis, Lymphadenopathies, Bronchiectasis	$\sqrt{1}/\sqrt{1}$	В-	54%
014A	CARD11 p.A817T	47	М	Respiratory, GI Salmonella sp	Enteropathy, Bronchiectasis	$\psi/\psi/\psi$	B-	18%
031A	CARD11 p.V659M	48	Μ	Respiratory, GI Campylobacter jejuni, E. coli	Enteropathy, Splenomegaly,	$\psi/\psi/\psi$	B+ SmB- Trnorm CD21lo	46%

Table 9, Clinical features of the patients with rare CARD11 variants.

One patient was found to be compound heterozygous for two rare germline missense variants. One variant inherited from the father (c.2759G>T; p.R920L) involving the L4 linker region between SRC homology 3 (SH3) and guanylate kinase (GUK) domains, and the other inherited from the mother (c.2066G>A; p.G689E) involving the PDZ (PSD-95/Dlg/ZO-1) domain. Those variants are absent in GnomAD and have not been previously reported. Both residues are evolutionarily conserved (GERP++ 0.21 and 0.23, respectively) and the substitutions are predicted to be deleterious (CADD 24.4 and 27.3, respectively).

The patient is a 44 year old female with a history of respiratory infections, recurrent herpes labialis, and one episode of zoster oticus, splenomegaly, and severe atopic dermatitis. Both parents are healthy, although the father has a history of sarcoidosis and the immunophenotype showed reduced memory B cells. Biallelic LOF variants of CARD11 are associated with a CID/SCID phenotype, while Dominant Negative monoallelic variants are associated with Immunodeficiency with Atopy (CADINS). The clinical phenotype of this patient (atopic disease, cutaneous viral infections, and respiratory tract infections) is evocative of CADINS. However, we identified two variants in *trans*. Moreover, the parents are clinically unaffected. We hypothesize that biallelic

hypomorphic LOF variants lead to a phenotype similar to CADINS. Further functional testing to assess the variant's functionality will be necessary.



Figure 10, Family pedigree and variant segregation of compound heterozygous CARD11 variants. Protein structure of CARD11 and mapping of the identified variants.

4.3.3 CD40LG

A missense variant in the CD40LG gene (c.107T>A, p.M36K) was identified. This variant is absent in large population databases and was reported as "likely pathogenic" in ClinVar. The residue is evolutionary conserved (GERP++ 4.51) and the substitution is computationally predicted to be deleterious (CADD 23.7). A missense variant in the same residue (p.M36R) has been already reported as pathogenic by Korthauer et al[31].

The variant was identified in a 42-year-old man who presented with laryngeal and facial mucocutaneous leishmaniasis. History was positive for visceral leishmaniasis at the age of 13, one episode of bronchopneumonia during childhood, and recurrent otitis. He had a brother who died of an unspecified lymphoproliferative syndrome. He had normal T CD4+ and CD8+, NK, and B lymphocyte counts. Serum IgG

(3.57 g/L) was reduced, with normal IgA (0.62 g/L) and IgM (2.30 g/L). There was no history of liver disease or neutropenia.

Switched memory B cells and T follicular helper cells were markedly reduced. The patient also had markedly reduced intracellular IFNy in T CD4+ cells activated with PHA. Surface expression of CD154 on activated T CD4+ cells was confirmed to be significantly reduced (27% with reference value between 40-80%).

Remarkably, the variant falls in the transmembrane domain of the protein where it replaces a non-polar amino acid with a charged one. Most pathogenic variants reported in CD40LG are missense variants in the extracellular domain or null alleles (stop-gain, frameshift). Few mutations in the transmembrane have been reported, and most of those insert charged amino acids in the protein sequences disrupting the protein membrane expression (p.M36R, p.G38R)[31,272]. Among those the reported phenotypes are often atypical, mild or mimicking CVID. The p.I33N variant was found in a patient with a diagnosis of CVID[213]. A small 6 nucleotides duplication leading to an in-frame insertion of a proline and alanine in position 40 was recently reported cause a mild hyper-IgM phenotype (c.116_121dupCAGCAC)[273]. Another case of "mild" hyper-IgM was reported with the p.H47Y which is the first amino acid of the extracellular domain.[274]

Single nucleotide variants in the transmembrane domain of CD40LG generate a protein with lower surface expression than wildtype, however, unlike large deletions or extracellular domain variants, they do not abolish the interaction with CD40. Hypomorphic variants in CD40LG can present with atypical features mimicking CVID and lacking the early onset of typical hyper-IgM syndrome.



Figure 11, Family pedigree and Sanger sequencing confirmation of the p.M36K variant in the patient and in a healthy sibling. Protein structure and mapping of the identified variant (red) and all other variants described in the literature in the transmembrane domain (TM). Expression of CD40L in the patient and healthy donors (HD) after PMA stimulation. Intracytoplasmic staining for IL-17 and IFNy after stimulation in the patient and a control.

4.3.3. CTLA4

Four patients were identified with mutations in CTLA-4, 3 novel missense variants, and one large deletion encompassing the CD28/CTLA4/ICOS locus. The CTLA4 includes 4 exons: exon 1 encodes the signal peptide, exon 2 the ligand binding and dimerization domains, exon 3 the transmembrane domain, and exon 4 the cytoplasmic tail. All pathogenic variants have been described in the extracellular and transmembrane portions of the protein.

The first novel missense variant involves the last base of exon 3 (c.567G>A, p.M189I) and it's predicted to affect the splicing site. This variant was not reported in any population database and is predicted to be deleterious (CADD 18.2). Splice site variants in the nucleotides downstream have been reported to cause CTLA-4 deficiency, in particular, a family with the c.567+5G>C variant was described by Kuehn et al.[194]. Moreover, the c.567+1G>A and c.567+3G>A variants have been associated with the phenotype in ClinVar. The patient is a 22 year old male, the mother did not carry the mutant allele and it was not possible to test the father as he died at a young age. The father was probably the carrier of the mutations, as he was diagnosed with CVID and had severe enteropathy and cytopenias. Additionally, the paternal aunt is diagnosed with selective IgA deficiency and enteropathy, though she was not tested for variant segregation. The patient meets the criteria for CID, as he has reduced naïve T CD4+ cells. He also has an elevated percentage of B CD21low cells (21%) which is compatible with CTLA-4 deficiency. Clinically he suffers from recurrent respiratory infections, several episodes of Molluscum contagiosum, and a chronic papulosquamous cutaneous rash that was diagnosed as psoriasis and Lichen ruber planus. The patient familial history, clinical phenotype, and variant analysis make this variant Likely Pathogenic.

The p.T147N variant lies between the homodimerization the ligand-binding domains. This variant was not reported in any population database, is a highly conserved amino acid and the substitution is predicted to be deleterious (CADD 25.9). Several pathogenic variants in the G146 residue have been reported (p.G146R, p.G146V, p.G146*)[195]. Moreover, a missense variant in the same position (p.T147A) is reported in ClinVar as associated with the phenotype. The patient is a 32 years old male with severe respiratory infections, thrombocytopenia, autoimmune hemolytic anemia, and autoimmune neutropenia, IBD-like enteropathy complicated with pyoderma gangrenosum, cholangitis with portal hypertension, and one episode of portal thrombosis. He also has a diffused cutaneous rash. It was not possible to test if the variant was transmitted or the *de novo*, notably the father is diagnosed with IBD. The immunological phenotype is characterized by markedly reduced naïve T cells with an oligoclonal expansion of aberrant lymphocytes, the B phenotype is characterized by a massive expansion of the CD21low B cells (81%). Given the current information, this variant is classified as Likely Pathogenic.

The third novel missense variant is p.S55N lying in the ligand-binding domain. This variant was not reported in any population database, the position is not conserved, and the variant is predicted to be tolerated by most prediction scores. The variant CADD score is 7.5 which is nevertheless higher than CTLA-4 MSC-CADD cutoff 0.001. A likely pathogenic variant (c.160G>A, p.Ala54Thr) is reported in ClinVar in the adjacent residue. The patient is 65 years old female with infection-only CVID and negative family history. The variant segregation in the family was not tested. She has mildly reduced naïve T CD4+ cells and mildly elevated B CD21low lymphocytes (11%). Data regarding the family segregation and the variant functionality are needed. Currently, the variant is classified as a VUS. In the fourth patient, a 225Kb deletion (chr2:204599508-204824322) was predicted in the 2q33 region containing the CD28 cluster, encompassing the CD28, CTLA4, and ICOS genes. The same patient is a carrier of two heterozygous variants in TNFRSF13B (TACI), it is not known if they are in *cis* or *trans*. The first variant is a highly deleterious nucleotide duplication causing a frameshift (c.204dupA, p.Leu69ThrfsTer12) mutation leading to an early stop codon. The second is a rare (GnomAD AF 7.974e-06) missense variant (c.554T>G,p.F185) in a conserved amino acid, and is predicted to be deleterious (CADD 25). The patient is 47 years old male with typical CVID, with respiratory infections, gastrointestinal infections by *C. jejuni*, refractory coeliac-like enteropathy, and splenomegaly. Both parents and his three children are healthy, however, the segregation of the mutations was not been tested.



Figure 12, Family pedigrees of patients with rare CTLA-4 variants. CTLA-4 protein structure and mapping of the variants. Predicted deletion of CD28, CTLA4 and ICOS based on reduction of observed over expected read ratio.

Patient	Variants	Age	Sex	Infections	Other conditions	lgG/A/M	EUROClass	T naive
009A	CTLA-4 p.M189I	22	Μ	Respiratory, Molluscum contagiosum	Enteropathy, Hepato- Splenomegaly, Psoriasis/Lichen ruber planus	$\psi/\psi/\psi$	B+ SmB+ CD21lo	7%

025A	CTLA-4 p.S55N	65	F	Respiratory		NA	B+ SmB- Trnorm CD21lo	14%
036A	CTLA-4 p.T147N	32	М	Respiratory, mycotic pneumonia	Cytopenias, Enteropathy IBD-like, Splenectomy, Portal thrombosis, Lymphadenopathies, Pyoderma gangrenosum	↓/↓/=	B+ SmB- Trnorm CD21lo	1%
022A	2:204599508- 204824322 (DEL CD28, CTLA4, ICOS) TACI p.L69fs p.F185C	47	Μ	Respiratory, Gl Campylobacter jejuni	Enteropathy, Splenomegaly	$\sqrt{1}/\sqrt{1}$	B+ SmB+ CD21lo	14%

Table 10, Clinical features of patients with rare CTLA-4 variants.

4.3.4 NFKB1

We identified a novel single-nucleotide duplication in NFKB1 leading to a frameshift (c.897dupT; p.D299fs) in the index patient of a family with a history of CVID over three generations. The variant was reported once in GnomAD (AF 0.000398%). Equivalent duplications in NFKB1 leading to the same frameshift were reported in several other kindreds with CVID[156,275]. The index is a 41 years old female with CVID complicated by granulomatous hepatitis and refractory coeliac-like enteropathy. She has greatly reduced B cells (Paris MB0, Freiburg Ib, EUROClass B-), 50% of her few B cells have a Transitional phenotype. The mother was affected by CVID and deceased from complications of her immunodeficiency. Her 15 years old son was diagnosed with selective IgA deficiency and Growth hormone deficiency and was found to carry the mutant p.D299fs allele. He has a milder reduction of the circulating B cells (Paris MB0, Freiburg II, EUROClass B+ SmB- Tr high CD21norm) but with a marked reduction of switch memory B cells and an expansion of the Transitional B cells. In summary, this variant is classified as Pathogenic.

4.3.5 NFKB2

One patient with the p.N447S was found. This variant replaces the asparagine at codon 447 with serine of the NFKB2 protein. The asparagine residue is moderately conserved and there is a small physicochemical difference between asparagine and serine. The variant CADD 12.49 is lower than the NFKB2 CADD-MSC cutoff 33. This variant was reported in GnomAD (AF 0.00415%), it is found at higher frequencies in subjects of South Asian descent (AF Popmax 0.0263%). This variant was not reported associated with immunodeficiency. The serine amino acid residue is found in multiple mammalian species, suggesting that this missense change does not adversely affect protein function. Most pathogenic NFkB2 variants are localized in the C-terminal portion.

The patient is a 50 year old male with infection-only CVID, the parents are consanguineous (first degree cousins) and there is no family history of CVID, pathogenic homozygous variants were not found. The parents were not tested. In summary, the available evidence is currently insufficient to determine the role of this variant in disease. Therefore, it has been classified as a VUS.

4.3.6 PIK3CD

We identified one patient with the p.R548W variant, replacing the arginine at codon 548 with tryptophan in the PIK3CD protein. The arginine residue is weakly conserved and there is a moderate physicochemical difference between arginine and tryptophan. The AF of this variant in GnomAD is 0.00295%. The CADD score is 26.7, above the MSC-CADD cutoff of 23.8.

Most patients with APDS have GOF mutations in the kinase domain (p.E1021K, p.E1025G, and p.R929C) or variants disrupting the inhibitory interaction between PIK3CD helical domain and the PIK3R1 nSH2 domain (E525K, E525A, and Y524N)[276]. The p.R548W variant is localized in the helical domain but it was not reported in subjects with APDS.

The patient is a 54 years old male with respiratory infections, bronchiectasis, hepatosplenomegaly, and chronic lymphadenopathies. The parents are healthy non-consanguineous, he also has one healthy daughter and one healthy brother. The variant segregation was not tested. The patient was also shown to carry the p.H159Y TNFRSF13C (BAFFR) variant. He had mildly reduced T CD4+ (540/mm3) with normal T CD4+ naïve, 38% of T CD8+ are CD57+, normal absolute B cells, with expanded CD21low (B+ SmB+ CD21lo), reduced IgM and IgG, absent IgA. In summary, the available evidence is currently insufficient to determine the role of this variant in disease. Therefore, it has been classified as a VUS.

4.3.7 PTEN

One patient was found to have the PTEN c.596T>C p.M199T variant. This sequence change replaces methionine with threonine at codon 199. This methionine residue is moderately conserved and there is a moderate physicochemical difference between methionine and threonine. This variant is absent from GnomAD and it wasn't reported associated with immunodeficiency (APDS-like) or PTEN-hamartoma syndrome. The CADD score is 10.53, which is higher than the CADD-MSC cutoff of 2.18. The variant is found in the C2 Tensin-type, contiguously to many reported pathogenic variants. However, there is no functional data to confirm the mutant function.

The patient is a 56 years old male, with respiratory infections, CMC, and splenomegaly. He was previously treated for non-Hodgkin lymphoma. There is no familial history of tumors or immunodeficiency, and familial segregation of the variant was not tested. The patient was also shown to carry the p.H159Y TNFRSF13C (BAFFR) variant. The patient has reduced naïve T CD4+ (9%) and B cells with nearly absent memory B cells and elevated transitional (63%) B cells (B+ SmB- Trhigh CD21norm). He has reduced IgG, IgM, and absent IgA.

The patient was found to have verrucous lesions on his hands that are under current dermatologic evaluation for possible trichilemmomas. In summary, given the patient phenotype and history and the variant data, it is classified as Likely Pathogenic.

4.3.8 TCF3

One patient had the c.9728C>T p.P310S variant in the TCF3 gene. One allele with this variant was reported in GnomAD (AF 4.882e-06). The CADD score is 23.5, higher than the CADD-MSC cutoff of 3.31. This variant is not reported in ClinVar and was not reported in patients with immunodeficiency.

The patient is a female 36 years old with respiratory infections, gastrointestinal infections by *C. jejuni*, recurrent cutaneous herpes simplex, splenomegaly and lymphadenopathies, vitiligo, and a history of non-Hodgkin lymphoma. There was no familial history of immunodeficiency. She had low IgG, IgA, and IgM, lymphopenia with very low T CD4+ naïve and almost absent B cells (0.5%). The few circulating B cells had prevalently a transitional phenotype, and they normally expressed the surface BCR immunoglobulins IgM and IgD. The variant p.E555K that was shown the be dominant-negative and associated with autosomal dominant agammaglobulinemia caused in all the described patients reduced B cells with bright CD19 expression and absent surface BCR[22]. In summary, the available evidence is currently insufficient to determine the role of this variant in disease. Therefore, the variant was classified as a VUS.

5. Discussion

The diagnosis of PIDs was historically founded on the pillars of clinical evaluation, family history, laboratory diagnostics, and immunophenotyping. Before the advent of NGS, the genetic diagnosis was mainly considered a research tool. Especially in the setting of prevalently humoral deficiencies, the clinical heterogeneity hindered the detection of the molecular defects underlying CVID and CVID-like disorders. In this study, we used WES to study a cohort of patients with a diagnosis of CVID. Interestingly, we identified pathogenic variants, variants of uncertain significance or mutations associated with CVID in half of the patients. Mutations in NFKB1, NFKB2, CTLA4, CD40LG, PIK3CD, PTEN, TCF3, and CARD11 were identified in 12 index patients and variants in TNFRSF13B (TACI) and TNFRSF13C (BAFFR) were found in 9 index patients. These findings confirm that WES is a useful diagnostic genetic approach to CVID.

The emerging genetic heterogeneity of hypogammaglobulinemias made Sanger sequencing impractical. Gene panels represent a faster and cheaper alternative to WES, however, the number of genes involved in PID diseases is growing by the day, making gene panels quickly obsolete. On the other hand, while WGS is destined to substitute WES in the long run, currently the small diagnostic improvement comes at much higher costs, analysis complexity, and storage requirements. WGS's main advantages are the ability to call SV and identify mutations in non-coding sequences.

Structural variants contribute to a significant part of monogenic defects, but they can be easily missed by WES. In our cohort, ExomeDepth identified a patient with a large deletion encompassing CD28, CTLA4 and ICOS, that was not identified by the WES. Besides, when a deleterious single nucleotide variant is found in a gene with biallelic inheritance, there is a possibility that a defect in the other allele is from a structural variant or a non-coding DNA mutation that is eluding WES detection. While WGS and CGH arrays are the preferred methods for SV detection, *in silico* CNV prediction algorithms from WES data improve the diagnostic yield without further costs.

Given that the definition of CVID implies the exclusion of other immunodeficiency syndromes, the use of NGS is becoming arguably essential as a correct genetic diagnosis can change the diagnosis and impact the clinical management.

We found in our cohort a patient carrying a CD40LG variant in the transmembrane domain. CD40LG deficiency causes X-linked hyper-IgM syndrome, however, hypomorphic variants have been seldomly shown to have milder clinical features, mimicking CVID. Another patient diagnosed with CVID with a similar variant was also identified in a large WGS study in PID patients[213]. Correctly identifying that the patient is affected by atypical hyper-IgM carries important clinical considerations, including prognosis and management.

One of the major challenges in the management of PID patients is to balance the necessity to use immunosuppressive therapies in subjects that are already immunocompromised. The discovery of gene defects leading to immunodysregulation enabled the adoption of targeted therapies. In our cohort, we identified 4 patients with defects in CTLA-4, three of those presented with multiple autoimmune diseases and often a family history characterized by relatives' deaths at a young age due to overwhelming autoimmunity. Abatacept is a CTLA-4-Ig recombinant fusion protein that was approved for the treatment of rheumatoid arthritis. Abatacept was successfully used in CTLA-4 deficiency, and other immunodysregulation syndromes affecting CTLA-4 endosomal trafficking like LRBA and DEF6.

We also identified two patients with a mutation in the PI3K pathway, in PIK3CD and PTEN respectively. The variants we identified are of uncertain significance and will need to be functionally validated by testing the phosphorylation of Akt and S6 kinase. Mutations leading to a hyperactivation of the PI3K/Akt/mTOR pathway are often associated with severe lymphoproliferation. Drugs targeting the downstream effector mTOR complex, like Sirolimus, can reverse the immunodysregulation. Moreover, specific p110 δ inhibitors, like Idelalisib and Leniolisib are in development and have shown promising results in clinical trials[277].

The main limitation of our study is the lack of functional validation of the variants of uncertain significance. This process requires an array of analyses geared to prove the clinical relevance of the variants. The main

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strategies include demonstrating an altered cellular expression, in vivo and in vitro functional assays, complementation assays with cellular or animal models. The complexity of this task can range from trivial to extremely complex and time-consuming. Usually, the simplest approach is proving an altered cellular expression, this is usually achieved with western blots and flow cytometry or indirectly by measuring RNA with qPCR. We demonstrated the deleterious effect of the CD40LG that we identified in one of our patients by measuring the surface expression of CD40LG by flow cytometry. However, surface protein expression can be inadequate, for example, CTLA-4 deficient patients and controls have often similar surface levels of CTLA-4. This can be caused by a conserved expression from the wild type, cellular protein dynamics (e.g. most CTLA-4 is stored in the endosomal compartment), predominant or restricted expression in a subset of cells (e.g. CTLA-4 in Tregs), or a variant not affecting the binding of the autoantibody used for detection but altering the protein function.

Assays measuring not only expression, but the function can overcome some of these limitations. For instance, CTLA-4 trans-endocytosis using Chinese hamster ovary cells transfected with CD80-GFP or recombinant CD80-GFP protein uptake was shown to be more sensitive for detecting deleterious single nucleotide variants CTLA-4 and LRBA mutations[278].

Demonstrating the pathogenicity of variants in genes that weren't previously associated with immunodeficiency requires a greater burden of proof. This process is hindered by the fact that often only single patient or kindred with the candidate gene mutation is identified. For this reason, large cohorts, multicentric studies, and collaborative efforts, like the "Undiagnosed Disease Network"[279], that help medical doctors, researchers, and patients to aggregate cases with similar variants play a pivotal role. Yet, single case studies, when backed up by a rigorous approach, have been proved to be valuable models that can significantly contribute to the understanding of Mendelian diseases[125]. Analyzing the variants segregation in the kindred in order to select those that are compatible with the Mendelian inheritance model is helpful to narrow down the number of candidate genes. Moreover, candidate genes should be prioritized using gene-level annotations assessing function and ontology, population genetics, phylogenetic conservation, and tissue expression. In summary, unbiased NGS techniques like WES and WGS moved the bottleneck from the discovery of variants to their functional validation.

We also identified 7 patients with variants in TNFRSF13B (TACI), the mutations were biallelic in two patients (1 homozygous, 1 compound heterozygous). These genetic variants have a low penetrance and are generally considered disease-associated rather than pathogenic. Three major groups can be identified according to the mutation type and location. The first are mutations affecting the extracellular ligand-binding domain, the prototypical variant in this group is the common p.C104R polymorphism. The second group includes nonsense and frameshift mutations leading to haploinsufficiency and probably account for the immunodeficiency observed in Smith-Magenis syndrome that is caused by 17p11.2 deletions. The third group

include missense variants in the transmembrane and intracytoplasmic domain, like the common A181E variants. Their mechanism of actions isn't fully elucidated, although mouse models support an haploinsufficiency mechanism[280]. A large multicentric Italian study including 189 CVID, 67 IgAD and 330 healthy controls identified 11% of CVID patients carrying TACI variants, 7% monoallelic and 4% biallelic. The frequency of TACI mutations was significantly higher in patients than in healthy controls, and only patients carried biallelic variants. However several monoallelic variants were found in healthy controls, and there was no significant difference in the frequency of the p.C104R variant[134]. This cohort also confirmed the theory that patients biallelic TACI variants actually have a more severe disease because haploinsufficiency leads to residual B cell responsiveness enabling autoimmune diseases.[136] In our cohort two patients have biallelic variants. The first patient with a biallelic TACI mutation is homozygous for p.C104R and has an infection-only phenotype. The second one is compound heterozygous for from a frameshift variant (p.L69fs) and a missense variant in the intracytoplasmic domain (p.F185C), clinically presenting with enteropathy. Nonetheless, two major caveats exist in this patient as he is the carrier of the CTLA4/ICOS/CD28 deletion, and it is not known if the two TACI variants are in *cis* or *trans*.

Immunophenotyping plays an important role in the diagnosis and prognostic stratification of CVID patients. Scarce data is available about immunophenotype-genotype correlations. In our cohort we couldn't identify any statistically significant phenotype-genotype correlation, largely due to the small number of patients for each gene. Moreover, there are no immunophenotypic features that are specific for any genetic variant. We confirmed some features that have been previously reported such as the elevated percentage of CD21low B cells in CTLA-4 deficiency, elevated Transitional B cells in the APDS patients and the low Tfh cells in the CD40LG patient.

In summary, our study confirmed the good diagnostic performance and clinical usefulness of WES in CVID and CID patients, as reported in other cohorts[281,282]. We showed that using in silico CNV prediction algorithms can further improve the diagnostic yield of WES. The unbiased approach of WES is essential to tackle the problem of genetic and phenotypic heterogeneity, as proven by the unexpected identification of an adult patient with a CD40LG defect.

Finally, our study contributed to the current knowledge of the field by identifying two previously unknown likely pathogenic CTLA-4 variants (p.M189I, p.T147N) and one novel NFKB1 variant (p.D299fs). We also identified several candidate VUS that will need to be validated with functional studies.

Our current analysis was limited to genes already described in association with PID diseases, but selected patients where family segregation is available are candidates for further studies. Furthermore, in some cases, other disease mechanisms are implicated like polygenic inheritance, somatic mutations, or epigenetic regulation.

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