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## PH.D. THESIS

# RARE INBORN ERRORS OF IMMUNITY: NEW INSIGHTS IN MOLECULAR BASIS, DIAGNOSIS AND TREATMENT


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**Background, summary of research activities and aims of the thesis**

Primary Immunodeficiencies (PIDs) represent a group of rare inborn errors of immunity due to defects in the development and/or function in various components of the innate and adaptive immune system (1).

PIDs are traditionally considered rare conditions, however, recent reports suggest that they are more common than previously believed, with an estimated prevalence of 2.3 per 100,000 persons (2). Overall, the incidence of PIDs varies from 1 in 600 to 1 in 500,000 live newborn, depending upon the specific disorder.

Recently, the International Union of Immunological Society (IUIS) Expert Committee on Primary Immunodeficiencies have proposed a new classification of these disorders into eight major categories, based on the primarily involved immune component and associated symptoms:

a. Immunodeficiencies affecting cellular and humoral immunity;
b. Combined immunodeficiencies with associated or syndromic features;
c. Predominantly antibody deficiencies;
d. Diseases of immune dysregulation;
e. Congenital defects of phagocyte number, function, or both;
f. Defects in intrinsic and innate immunity;
g. Autoinflammatory disorders;
h. Complement deficiency

Among all the immunodeficiencies, antibody deficiencies are the most frequent and comprise approximately 70–75% of all PIDs (3).

These disorders are characterized by a wide range of clinical symptoms, including an increased rate and severity of infections, sometimes with accompanying autoimmune disease or auto-inflammatory diseases, allergy and malignancy (4).

Early diagnosis of PID is useful in order to prevent significant disease-associated morbidity and mortality. However, to date the diagnosis of a specific PID based on the

analysis of the clinical and immunological phenotype remains difficult and a considerable delay, between the onset of the symptoms and diagnosis, is often reported.

Furthermore, expressivity and penetrance of each disorders vary widely, even among family members with the same specific mutation. These observations suggest that likely other genetic, epigenetic, and/or environmental factors may contribute to the clinical disease phenotype (4).

In the last years, T cell receptor excision circles (TRECs)–based newborn screening has been implemented in several countries for neonatal detection of some T-cells deficiencies such as SCIDs or profound T cell lymphopenia (5).

Compared with patients identified by the clinical features, patients identified through newborn screening programs, can receive an early and accurate diagnosis by one month of life and then undergo to curative treatments such as hematopoietic stem cells transplantation (HSCT) or gene therapy, before the occurrence of severe complications. This results in a significantly improved outcome (6, 7).

Until 2010, the traditional approach to PIDs has included Sanger sequencing of candidate genes, single nucleotide polymorphisms (SNPs), linkage analysis and an array of analytic and functional tests, including the proliferative response to mitogens, flow-cytometry, cytotoxicity assays, neutrophil function tests etc, that can provide a detailed immunological characterization of the patients (8). In the last years, the method for the classification of the immune cells by their surface protein expression patterns through flow-cytometry is rapidly evolving, thus permitting to better define the phenotype of each immune cells and to better understand their biologic role in patients with several immune disorders (9).

Overall, the number of genetically defined PIDs has increased significantly over the past 20 years (3), and more than 300 disorders have been identified up to now, thanks to the availability of positional cloning and, more recently, massively parallel (next-generation) sequencing technologies. Only in the last years, more than 30 new genes have been identified (3). NGS technologies are revolutionizing the discovery of genes in which variants can cause rare Mendelian diseases (10), replacing the gene by gene strategy with the possibility to sequence a very large panel of genes or the whole genome. This approach is particularly promising for the diagnosis of rare pediatric disorders such as PIDs characterized by a strong clinical and genetic heterogeneity.

The majority of the new described phenotypes results from complete or partial loss of function of the gene product, but more recently, increase number of diseases due to a gain of function (GOF) effect have been described (11). In some cases, CARD11 and STAT1 for example, there are both autosomal dominant GOF and autosomal recessive loss of function (LOS) variants, and these different modes of inheritance can lead to different functional consequences and different immunological and clinical phenotypes (3).

In this perspective, the characterization of patients with very complex phenotypes using NGS technology is dramatically increasing our understanding of the genetic basis and of the pathogenic mechanisms of PIDs.

Furthermore, the identification of a link between newly identified genes and the specific functional abnormalities resulting therefrom, is opening the door to targeted therapies for optimal clinical management of the patients affected by immune disorders. This approach represents one of the central components of precision medicine (8).

Precision medicine not only has the real opportunity to benefit patients with PIDs, but it might also increase understanding of the immune-pathogenesis of a variety of PIDs. As has been the case for a number of therapeutic advances in human disease management that include HSCT and gene therapy, PIDs represent a unique group of disorders that will continue to be in the forefront of defining new and targeted immunomodulatory therapies and help define unique therapeutic approaches in the evolution of precision medicine (8).

During my PhD program in "Clinical and Experimental Medicine" (XXX Cycle, years 2014-2017) I contributed to the evaluation of the potential benefit of betamethasone on neurological symptoms and quality of life of patients affected with Ataxia-Telangiectasia. In particular, in a multicenter study, performed with a blind evaluation procedure, we have tried to define the minimal effective dosage of betamethasone in the perspective of an occasional usage of the drug, thus preventing the occurrence of side effects in A-T patients.

Moreover, I participated in the clinical, functional and molecular characterization of patients with well-known form of PIDs and in the implementation of new approaches for the clinical management of such patients. In particular, this thesis was focused on the following lines of research:

a. Phenotypic characterization and identification of novel pathogenetic aspects related to PIDs, with attention to recent discovered gene;

b. Characterization of a novel immunodeficiency whose hallmarks are represented by high IgM levels, impaired B-cell homeostasis and cancer susceptibility,

c. Phenotypic, immunological and molecular characterization of new forms of PIDs identified through next generation sequencing, Sanger sequencing method and array-CGH;

d. Better definition of the malformative spectrum, including lung and ear-nose-throat disorders in children with 22q11.2 deletion syndrome and their role as risk factor for the pathogenesis of respiratory infections;

e. Characterization of skin and skin annexes abnormalities associated to PIDs and definition of the role of T independent and B cell immunity in the susceptability to infenctions in patients affected with Hypoidrotic Ectodermal Dysplasia with Immunodeficiency;

f. Study of the functional role of FOXN1 Transcription factor in T-cell ontogeny;

g. Rare genetic syndrome involving immune system paying particular attention to SCID, and 22q11.2 deletion syndrome.

# CHAPTER I

## "Minimum Effective Betamethasone Dosage on the Neurological Phenotype In Patients With Ataxia-Teleangiectasia: A Multicenter Observer-Blind Study"

## Introduction

Ataxia-telangiectasia (A-T) (MIM 208900) is a rare recessive neurodegenerative disease that results from inactivation of the A-T Mutated (ATM) gene encoding for a protein kinase (12). The disorder affects 1 in 40,000 to 300,000 live birth worldwide (13). Accordingly to the European Society for Primary Immunodeficiency (ESID), in Europe, 410 living A-T patients are currently listed.

The clinical picture of this condition is very complex and variable. The severity of the pulmonary, immunological and neurological phenotype varies widely between patients and it is related to the severity of the underlying mutations and any residual ATM kinase activity. It has been recently suggested that the name A-T should be replaced by "ATM syndrome" (14).

The ATM gene is large, spanning 150 kb of genomic DNA and encoding an ubiquitously expressed transcript of approximately 13 kb, consisting of 66 exons, giving a 350 kDa protein of 3056 amino acids. ATM is predominantly a nuclear protein, but it surprisingly displays prominent cytoplasmic localization in mouse Purkinje cells (15)

A-T is considered the prototype of the DNA-repair defect syndromes. In fact, ATM represents the central component of the signal-transduction pathway responding to DNA double-strand breaks (DSBs) caused by ionizing radiation (IR), endogenous and exogenous DNA damage agents. In response to DSB formation, ATM, and further DNA-repair and cell cycle checkpoint proteins are activated, leading to cell cycle arrest and DNA repair (16). The activation of ATM kinase involves autophosphorylation of serine 1981 of the protein and subsequent dissociation of inactive ATM dimers into active monomers. Chromosomal DSBs are potentially one of the most dangerous forms of DNA damage that, if left unrepaired, can result in chromosomal aberrations, deletions, or translocations. Such abnormal process could also account for the high incidence of chromosomal rearrangements involving primarily the chromosomes 7 and 14, corresponding to the sites of the immune system genes. Defects in DSBs repair are linked to cell death and tumorigenesis .

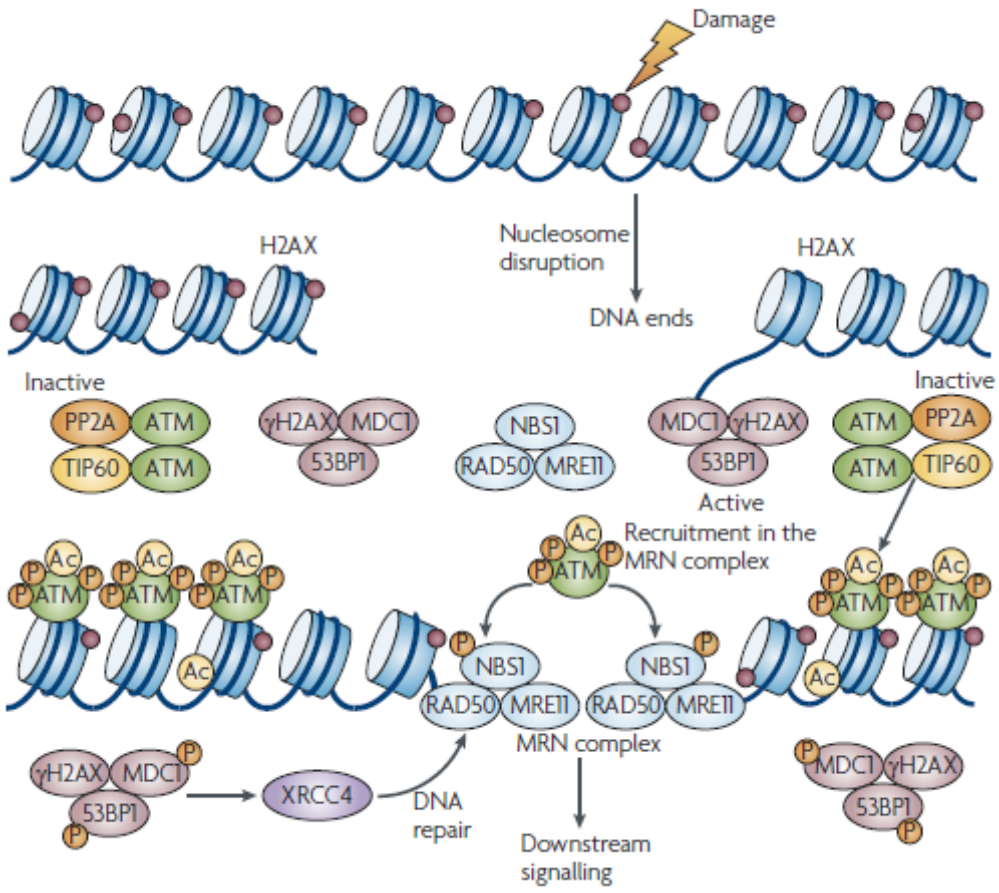


**Figure 1.** Activation of ATM as a consequence of a DSB, involves the recruitment of the MRE11–RAD50–NBS1 (MRN) complex to the break and also the recruitment of ATM to regions that flank the break. ATM phosphorylates p53 and other substrates. ATM is then recruited to the site of the break by the MRN complex and phosphorylates members of the complex and other downstream substrates (Lavin MF 2008).

Evidence exists that ATM exerts additional functions in the cytoplasm independent of its role in the DNA damage response, such as participation in the autophagy pathway (17). More recently it has been documented that ATM is also present in the peroxisomes, cytoplasmic vesicles and mitochondria (18-20). Autophagy alterations have been implicated in several chronic nervous system disorders, such as proteinopathies (Alzheimer's, Parkinson's, Huntington's diseases) and acute brain injuries, whose hallmarks are organelle damage, synaptic dysfunction and neuronal degeneration. Autophagy is a constitutive lysosomal catabolic process during which, cytoplasmic components, damaged proteins and entire organelles are degraded and recycled to generate building blocks for anabolic processes. Autophagy, known originally as an adaptive response to nutrient deprivation in mitotic cells, including lymphocytes, is now recognized as an arbiter of neuronal

survival and homeostasis in that neurons are post-mitotic cells, which require effective protein degradation to prevent accumulation of toxic aggregates (21-23).

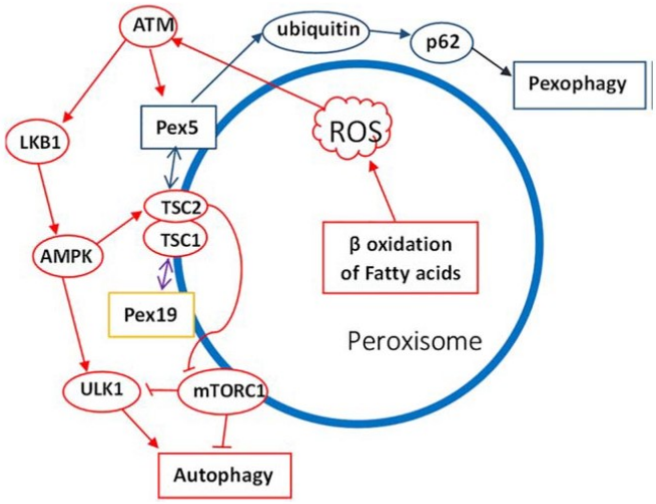


**Figure 2.** ATM signaling pathway at the peroxisome in response to ROS. Activation of AMPK leads to the phosphorylation of ULK1 kinase, which is essential for autophagy (Choi KR, 2017)

ATM is also involved in immune cell maturation, which requires gene rearrangements and therefore leads to DSBs. However, ATM deficiency does not result in a profound block in lymphocyte development. Differently, VDJ recombination may be affected. In B cells, this altered process leads to a defect in class switch recombination (CSR) from IgM to other classes, demonstrating the central role of ATM in class switching (24).

**ATM and neurodegeneration**

In the nervous system, defective DNA repair leads to neurodegeneration. Several mechanisms by which deficient DNA repair in neurons triggers their apoptosis have been proposed (25). The post-mitotic status of differentiated neurons may make them more vulnerable to DNA damage than cells in the active proliferation status. Genetic deficiencies in enzymes involved in the DNA repair process can induce neuronal apoptosis or make neurons more sensitive to further genotoxic stresses. Even though the progressive neurodegeneration is a common hallmark of many progressive

neurologic syndromes, all sharing a defective DSBs responses in their pathogenesis, the disease-specific differences in the onset and course of neurodegeneration likely reflect selective DNA repair requirements in the different areas of the nervous system (25).

There are two major DNA repair pathways for the DSBs damages. Homologous recombination repair (HR) is an important process mainly during early embryogenesis, where proliferation is at its maximal expression leading to the development of stem cells and progenitors. This complex machinery requires genomic integrity. Non-homologous end-joining (NHEJ) recombination repair is active mainly in the brain. In the mature nervous system, a different pathway repairs DNA single-strand breaks (SSBs) (26). In the nervous system, ATM signaling appears to function predominantly in immature and post-mitotic neural cells, suggesting that ATM responds to DNA DSB utilizing NHEJ. In the absence of ATM, neurons survive and populate the Purkinje neuron layer and only later they degenerate as a result of DNA damage experienced during development. This would explain the reason by which ATM is an important signaling molecule only in a selective region of the nervous system (27).

Neither the normal function of ATM in the nervous system nor the biological basis of the degeneration in A-T is known. The pathological features of A-T are predominated by the selective Purkinje cell depletion and granule neurons with partial thinning of the granule cell layer. Progressive atrophy of the cerebellar cortex is a hallmark of A-T, characterized by the appearance of abnormal Purkinje cells in the molecular layer of the cerebellum with abnormal smooth dendrites, reduced arborizations and finally, ectopic cells (26, 28-29).

Although ATM is known to be neuroprotective in the tissue undergoing oxidative stress and apoptosis, the molecular mechanisms of its function in the nervous system are uncertain. Since oxidative stress causes DNA damage it is difficult to make a clear distinction between alterations in cellular signaling induced by oxidative stress and DNA repair deficiency. So further works are necessary to unravel the role of ATM in response to oxidative stress (30).

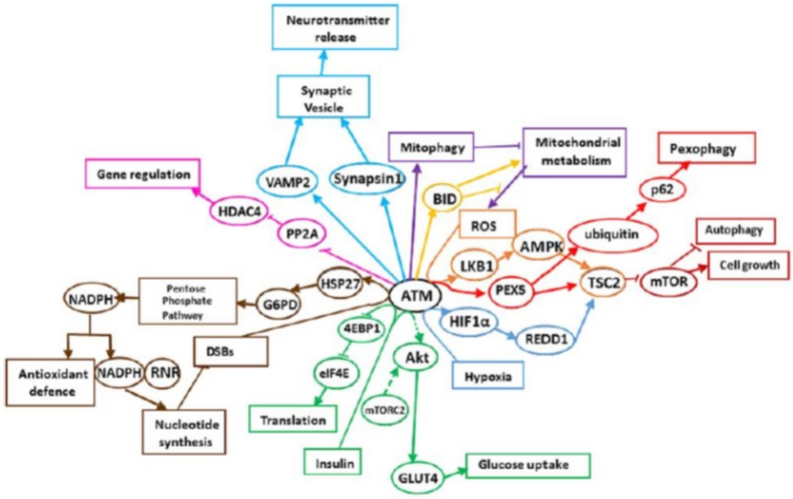


**Figure 3.** Network of cytoplasmic ATM signaling in cellular homeostasis (Choi KR, 2017).

## Clinical phenotype of A-T

### *Neurological phenotype and cutaneous manifestations*

A-T belongs to a group of early onset childhood ataxias, which affect children and young people, in whom the neurological dysfunction worsens over the time, eventually confining the patients on a wheelchair by the adolescence.

Patients with A-T typically present with signs of progressive neurological dysfunction, characterized by cerebellar ataxia and uncoordinated movements with onset between age one and four years, associated with deterioration of gross and fine motor skills occurring by approximately four years of age (31). A-T patients have normal mental skills and IQ tests, even though some deficts in nonverbal memory, verbal abstract reasoning and calculation and executive function may be detected.

Ocular abnormalities are an early feature of A-T and include oculomotor apraxia, which may be a main sign helpful in the early recognition of the patients, nystagmus, and photophobia (32). Oculomotor apraxia associated to slurred speech often leads to impassive facies. Reading difficulties are often observed in patients with A-T due to abnormalities of accommodation and eye

movements. In addition, a peripheral axonal neuropathy may be found and leads to decreased deep tendon reflexes.

Being almost invariably disabling, this illness has severe impact on the patients' quality of life (QoL) and psychosocial skills, significantly limiting the ability to perform tasks of daily life, restricting autonomy and social participation.

The second major clinical manifestation of A-T is represented by oculo-cutaneous telangiectasias, occurring later between two and eight years of age. Other dermatological features include hypo/hyperpigmentation, cutaneous atrophy, partial albinism, premature graying of hair, scleroderma-like lesion. Cutaneous granulomatosis has also been recently described in children with A-T (33)

**Immunodeficiency and pulmonary complications**

A variable immunodeficiency, affecting the humoral and cellular systems, is present in 60 to 80% of patients with A-T. The immunodeficiency is variable and does not correlate well with the frequency, severity or spectrum of infections.

The most common humoral defects are low or even absent IgA, IgE, and IgG2 serum levels, inconstantly associated with impaired antibody responses to vaccines. A few patients may also have elevated IgM serum levels, thus suggesting an Hyper IgM syndrome.

The most common defects of the cell-mediated branch are lymphopenia with low CD4 counts resulting in reversed CD4/CD8 ratio and impaired lymphoproliferative responses to common mitogens and antigens. A defect in recombination or DNA rearrangement may explain the defects in both T and B cell differentiation. However, unlike most immunodeficiency disorders, severe infections are uncommon in A-T and the spectrum of infections in individuals with A-T does not comprise opportunistic infections but predisposition to sino-pulmonary infections (34, 35).

As for pulmonary complications, three major lung disease phenotypes have been recognized up to date:1) recurrent upper and lower respiratory tract infections (RTIs), which in turn can lead to bronchiectasis 2) lung disease associated with dysfunctional swallow and inefficient cough due to the neurodegenerative deficit; and 3) ILD/pulmonary fibrosis (36, 37).

The frequency and severity of infections correlates more with general nutritional status than with the immune status.

Morbidity and mortality in these patients is significantly related to pulmonary manifestations, as recurrent sinopulmonary disease and bronchiectasis, interstitial lung disease and pulmonary fibrosis (38).

***Predisposition to cancer and chromosomal instability***

The prevalence of cancer in A-T is 10-30%, representing the second cause of death (39). Leukemia and lymphoma account for about 85% of malignancies. Most leukemia are of T-cell origin, while lymphomas are usually of B-cell type (40). Other solid tumors include ovarian cancer, breast cancer, gastric cancer, melanoma and gonadic cancer. The predisposition to develop tumors is best explained by genome instability due to altered repair of double-strand breaks. An increased risk of cancer, particularly of breast cancer, has also been described among A-T heterozygotes (41). In a careful analysis, heterozygote carriers (1% of the population) had an increased mortality rate from the second decade, with a progression with the age, mainly due to cancer, particularly breast cancer, or ischemic heart disease.

Patients with A-T also show an increased sensitivity to ionizing radiations. In vitro, radiosensitivity is expressed as reduced colony forming ability (CFA) following exposure to ionizing radiations or radiomimetic chemicals (42).

***Endocrine dysfunction and other phenotypic features***

The most common endocrine manifestations are: growth failure, hypogonadism and insulin-resistant diabetes mellitus (43, 44) Other clinical problems may include orthopedic manifestations, sleep disturbance and liver abnormalities.

Expectancy life is about 25 years, even though a longer lifespan has been reported in patients with A-T variant, which may reach the 4th-5th decade of life. Both the type of ATM mutation and residual kinase activity may contribute to the survival of A-T patients (38).

**Diagnosis and treatment**

Diagnosis of A-T relies on clinical phenotype, family history and is usually supported by laboratory findings that include: elevated serum α-phetoprotein (AFP); immunological deficiencies; cerebellar atrophy at MRI; chromosome analysis (7;14 translocation) on lymphocytes of peripheral blood; in vitro radiosensitivity assay; absent or markedly decreased intracellular ATM protein levels by

Western Blotting; deficient phosphorylation of ATM substrates through ATM serine/threonine kinase activity. Finally, the diagnosis of A-T is confirmed by molecular genetic analysis of ATM gene (45, 46). As NGS becomes standard clinical practice for patients with atypical signs, it is likely that more people with mild form of A-T will be diagnosed. Finally, several infants have been diagnosed as A-T thanks to SCID newborn screening test programs in combination with NGS.

Unfortunately, there is currently no treatment for A-T except for supportive therapy of secondary symptoms. The treatment of A-T remains based both in medical management of immunodeficiency, sinopulmonary infections, neurologic dysfunction and malignancy, both neurorehabilitation (physical, occupational, and speech/swallowing therapy; adaptive equipment; and nutritional counseling). In particular, A-T is a multisystem disease requiring intervention to: halt progressive neurodegenerative changes; reduce the risk of tumours; prevent severe infections due to the immunodeficiency; ameliorate respiratory functionality.

Unfortunately, no effective disease-modifying therapy is presently available for any of the major problems of the syndrome. There is no cure for the progressive neurodegeneration, but medications directed to partially control drooling and tremors. Attempts to relief the neurological symptoms of A-T have so far been made with L-DOPA derivatives or dopamine agonists to correct basal ganglia dysfunction

Since incorporation of myo-inositol into phosphoinositides, as well as free myo-inositol content, is low in some A-T fibroblasts and phospholipid metabolism is less active in A-T as compared to normal cells, as well, a potential effect of myo-inositol has been postulated on neurological and immune functions in A-T (47). However, although some promising results were observed in certain immune cells in a few A-T children (www.treatAT.org) in a first A-T clinical study, the sample size was not large enough to allow a conclusive interpretation of the data.

Antioxidant therapies were expected to simultaneously slow the progression of the neurological deterioration and to reduce the risk of cancer, as well. At a preclinical level, administration of the antioxidant 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (CTMIO) to *Atm*-deficient mice reduced the rate of cell death of Purkinje cells and enhanced dendritogenesis to wild-type levels, suggesting a protective role against neurodegeneration. Recent evidence also indicates that CTMIO dramatically delays the onset of thymic lymphomas in ATM-/- mice (48).

Despite these encouraging preliminary results, in humans, only a modest improvement has been achieved with antioxidant agents. A second group of antioxidants molecules, alpha-lipoic acid

and a poly ADP-ribose polymerase (PARP) inhibitor, nicotinamide, has been tested in a randomized, double blind, double dummy trial. Two oxidative stress markers, levels of urine total alkanes and serum fast oxygen reduced adsorbance capacity (ORAC), improved in comparison with the baseline, in particular when a combined therapy with both the antioxidants was. It is noteworthy that a trend toward increased lymphocyte counts was observed when subjects took both drugs, even though the difference did not reach a statistical significance. Concerning the study multiple neurologic parameters evaluated in this trial (quantitative evaluation of tremor, tone, saccadic latency and A-T index score) and pulmonary function through spirometry, no positive significant change was found in any of them evaluated parameters (49).

Overall, all attempts with anti-oxidant agents failed to halt the progressive nature of the disease. As for the correction of ATM gene function by read-through of premature termination codons, Lai et al. employed aminoglycosides to achieve read-through expression of functional ATM protein (50). In principle, these drugs bind to the RNA decoding site, inducing a conformational change that compromises the integrity of the codon–anticodon proofreading and allowing translation through an otherwise terminating codon. Gatti's group showed that geneticin and gentamycin produced detectable 'readthrough' ATM protein, as well (51). This methodology is very promising; however, it requires the use of aminoglycosides that are toxic to cells and humans at concentrations that would be effective for read-through. Further attempts have been made with antisense morpholino oligonucleotides (AMOs) to redirect and restore normal splicing in the ATM gene, by targeting aberrant splice sites and enabling expression of normally spliced full-length ATM mRNA. However a number of issues need to be addressed before it could be employed as a human therapeutic.

Symptomatic treatment can greatly improve the poor quality of life of these patients and prevent complications that could lead to death. Treatment of the symptoms of cerebellar ataxia should be symptom-focused (imbalance/incoordination/dysarthria, cerebellar tremor) and monitored with a few simple reproducible and semiquantitative measures of performance.

In the last years the potential benefits of glucocorticoids (GCs) for A-T have been considered. Several clinical observations documented a clear cut beneficial effect of such therapy that was inversely correlated with the extent of cerebellar atrophy (52-54). This beneficial effect was also inversely correlated with the age of the patients (55).

In addition, a beneficial effect was also documented at very low dosages of drug as 0.01 mg/kg/day of oral betamethasone (53). Of note, this effect was strictly drug dependent, in that the

drug withdrawal paralleled the worsening of the neurological signs (52). Furthermore, during the short steroid trial, a paradoxical effect on the proliferative response to mitogen stimulation was documented, differently to what expected on the basis of the drug-induced immune suppression, suggesting a direct effect of betamethasone on the intimate altered pathogenic mechanism in A-T (53).

As for the mechanism underlying this effect of corticosteroids on neurological symptoms in A-T, any definitive explanation is currently available. The interaction with specific receptor proteins in target tissues have been shown to regulate the expression of corticosteroid-responsive genes. Several lines of evidence indicate that GCs have remarkable effects through both non-genomic and genomic mechanisms, the latter well documented also in neural system. The classical genomic mechanism of GCs action is cytoplasmic glucorticoid receptor (GR) mediated. GCs bind and induce GR activation, followed by the GR translocation to nucleus and subsequent binding to glucocorticoid responsive element (GRE), thus modulating the transcription of a variety of genes including glucocorticoid-induced leucine zipper (GILZ). GILZ is known as a marker GCs transcriptional activity, rapidly induced by GCs, able to regulate T lymphocytes activity, including T cell survival. An alternative explanation of the beneficial effect of betamethasone in A-T could be a potential activity of this molecule as an antioxidant. This mechanism was addressed by in a pilot study of our group, where intracellular glutathione levels, reactive oxygen species (ROS) production, and lipid peroxidation were measured in A-T patients receiving betamethasone (55).

A marked reduction, but drug-dependent, of ROS levels in the more drug responsive patient was noted. It is noteworthy that the neurological improvement was observed by 1 week of treatment. The previous observation that older patients failed to respond suggests that a threshold level of Purkinje cell numbers or other cerebellar hard-wiring may be a prerequisite for successful steroid therapy in A-T (55, 56).

Thus, on the basis of very limited studies, it is mandatory that further evidence have to be gathered as to their potential role as disease-modifying agents.

## Aims

Aim of this multicenter study, performed with a blind evaluation procedure, is to define the minimal effective dosage of betamethasone in the perspective of an occasional usage of the drug, thus preventing the occurrence of side effects.

## Methods

### Patient selection

Patients included in this study (9 subjects within 8 families; 4 males), 4 to 25 years of age, received a diagnosis of A-T according to the European Society for Immunodeficiencies (ESID) criteria, confirmed by ATM gene sequencing (Table I). The study was approved by the Institutional Ethics Committee (Ethical Committee for Biomedical Activities of Federico II University), conducted in accordance with the ethical principles of the Declaration of Helsinki, and the local laws of the countries involved. An informed consent was obtained from all patients (or parents/guardians for pediatric subjects) during the screening visit**.** Standard clinical assessment was performed and data were collected through case report form (CRF), including age, gender, age at onset of first symptom, disease duration, presence or absence of the following findings: cerebellar ataxia, apraxia, dysarthria, resting tremors, previous history of recurrent or severe infections. Patients enrolled were required to be $\geq 3$ years old, to have a score $\geq 10$ on the Scale for the Assessment and Rating of Ataxias (SARA) and levels of CD4+ lymphocytes $\geq 200/mm^3$ at the screening visit. Any steroid assumption and/or concomitant use of other agents acting on the central nervous system were prohibited for a period of at least 30 days prior to start of treatment with betamethasone and throughout the study. Permitted medications included any drug needed to treat concomitant infectious events or any adverse effect associated with steroid administration, if any. Exclusion criteria from the study included: current or previous neoplastic disease, history of severe impairment of the immune system, chronic conditions representing a contraindication to the use of steroid drugs, participation in any other investigational trial within 30 days before the screening period.

### Trial design and procedures

This 8-month multicenter study was aimed at the evaluation of the efficacy and safety of betamethasone at progressively increasing dosages of 0.001, 0.005, and 0.01 mg/kg/day in one daily dose, in patients with A-T. The enrollment was performed at 3 Centers of the Italian Network of Primary Immunodeficiencies (IPINET), namely Federico II University of Naples, Spedali Civili of Brescia and University of Milan. Visits were scheduled at the screening, baseline (T0), every 60 days during the treatment period (T1-T3), and at the end of tapering and wash out period (T4). Patients started study medication at T0. At beginning of T3 patients de-escalated betamethasone of 25% every 5 days (Figure 4). In order to have a surrogate marker of compliance to the treatment, serum levels of ACTH and cortisol were monitored at each time-point. Cerebellar atrophy score was calculated as follows: a score of 0 =no cerebellar atrophy; 1 = no cerebellar atrophy but moderate pontocerebellar angle cisterns enlargement; 2 = moderate atrophy involving mostly both superior and inferior portion of the vermis and, at a lesser extent, the cerebellar hemispheres with moderate enlargement of periliquoral spaces; 3 = severe atrophy of superior portion of vermis and moderate atrophy of inferior part of vermis; severe atrophy of superior and lateral portion of cerebellar hemispheres and moderate atrophy of inferior hemispheres; 4 = global and severe atrophy of the superior and inferior part of vermis and the whole cerebellar hemispheres with marked fourth ventricle enlargement (Broccoletti T 2009 et al).

At each Center, a two-physicians treating and assessing model was used; the treating physician was responsible, in an open setting, for drug administration, recording of adverse events, and safety assessment. A well experienced neurologist performed the neurological evaluation. Each neurological evaluation, through a SARA scale (see appendix E1 on the Neurology Web site at http://www.neurology.org/cgi/content/full/66/11/1717/DC1), which allows to quantify ataxia severity on a scale from 0 (optimal) to the maximal score of 40, was videotaped at each Center. At the end of the study (T4), the videotapes of the patients, containing the 5 visits evaluation, were collected at the Scientific Coordinator Center (SCC), where a stochastic assembly of 2 min sequences for each individual SARA parameter was performed. Subsequently, the sequences were evaluated by a Neurology Evaluators Committee (NEC), consisting of three independent paediatric neurologists with experience in the management of A-T patients, that scored them separately and in blind according to SARA guidelines. Before assessment, observers were not informed about any children's characteristics.

For the evaluation of QoL, children or their parents/tutors were asked to complete at each time-point the Italian version of the Children Health Assessment Questionnaire (CHAQ). The

CHAQ assesses 8 functional areas (dressing, arising, eating, walking, hygiene, reaching, grip and activities) with a score ranging from 0 to 3 (0 = without any difficulty; 1 = with some difficulty; 2 = with much difficulty; 3 = not possible). The question with the highest score determined the score in that functional area. If aids or devices were used or help was needed to complete tasks in a certain area, a minimum score of 2 was recorded for the corresponding functional area. The scores of each of the 8 functional areas were averaged to calculate the CHAQ-disability index (CHAQ-DI), which ranges from 0 (no or minimal physical dysfunctioning) to 3 (very severe physical dysfunctioning). The CHAQ also allows to assess the presence of pain through a visual analogue score. However, the evaluation of this category was not applicable to our cohort of A-T patients.

The final evaluation of all safety and efficacy parameters and the statistical analysis was performed at the end of the study at the SCC.

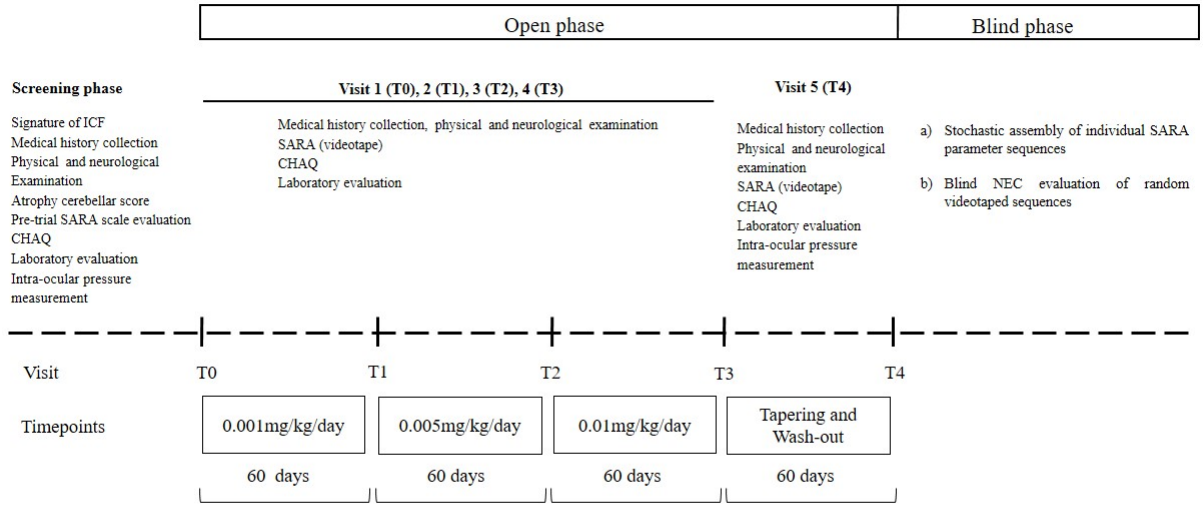


**Figure 4.** The flowchart illustrates the schedule of enrolment, interventions and assessment; ICF, informed consent form; SARA, scale for the assessment and rating of ataxia; CHAQ, childhood health assessment questionnaire; BUA, broadband ultrasound attenuation.

## Trial outcome measures

The primary objective of this study was to determine the efficacy of the treatment with different doses of betamethasone in A-T, as assessed by change from baseline in SARA scale. Patients were

defined as responder if a drop of at least 3 points of SARA score was documented at almost one of the time-points, while they were defined as partial responders if the drop was ≥1.5 and < 3 points. The cut off of 3 points of SARA score was considered informative on the basis of the previous study of Broccoletti et al. Furthermore, each SARA item was considered improved (or worsened) if a change of at least one point was documented by two independent evaluators.

The secondary objective was to determine the safety of the drug administration by reporting adverse events through the collection of the medical history, including medical events and changes in concurrent medications, physical examination, body weight and blood pressure monitoring ,a 12-lead electrocardiogram (ECG), the results of hematology and blood chemistry tests, including blood glucose and hemoglobin A1C levels, neutrophil counts, serum concentration of electrolytes, calcium and phosphorus, intra-ocular pressure measurement, at different time points. The severity of the adverse event was graded as mild (minimal or no treatment required and no interference with the patient's daily activities); moderate (low level of inconvenience or concern, might need treatment and cause some interference with functioning); severe (patient's daily activities interrupted and systemic drug therapy or other treatment needed, usually incapacitating); and life-threatening (immediate risk of death).

The tertiary objective was the QoL evaluation. An improvement was considered when a reduction of at least 15% in the disability index or in at least two CHAQ categories was observed.

## Statistical Analysis

Efficacy analysis was performed on the intent-to-treat (ITT) and per protocol (PP) populations. The PP population was defined as patients who completed the study, and had no major protocol violations. Descriptive analysis of the patient population was evaluated by calculating means and standard deviations for continuous variables and frequencies and percentages for all discrete variables. To assess the reliability of SARA score, the evaluation of intra-class correlation coefficient (ICC) was performed (Supplementary Table 1).

## Results

### Patients and baseline characteristics

Demographic characteristics are reported in Table 1. The median time since diagnosis of A-T and

range were 8.4+/-5.7 and 2.6-22 years, respectively. Median age at the disease onset and range were 29+/-11.5 and 18-48 months. All the patients were compound heterozygotes for ATM gene alteration**.** In all patients, ATM was not expressed. Patients 7 and 9 were siblings. All the patients had cerebellar ataxia. At the enrolment, 2 patients (P1 and P4) had grade 3-4 cerebellar atrophy; 3 patients (P5, P8 and P9) grade 2; 1 patient (P7) grade 1. In 2 further subjects (P3 and P6) no atrophy could be detected (grade 0). In a further subject (P2), data on a recent brain MRI was not available, but a previous evaluation revealed a grade 2 score. Only P6 was receiving Ig replacement therapy.

**Table 1** Demographic and clinical data of the cases enrolled in the study

| | | | | | Neurological phenotype | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Patient | Sex | Age at examination, years | Disease duration, years | Cerebellar atrophy score | Wheel chair confinement | Resting tremor | Dysarthria | Apraxia | Previous multiple/severe infections | Mutations |
| 1 | M | 13 | 11 | 3-4 | + | + | + | + | + | 97delC/2113delT, heterozygous |
| 2 | F | 12 | 8 | 2 | + | - | + | + | - | 381delA/6679C>T, heterozygous |
| 3 | M | 4 | 2.6 | 0 | - | - | - | - | + | c.2376G<A/792del42, heterozygous |
| 4 | F | 24 | 22 | 4 | + | + | + | + | + | 8629insC/8977C>T heterozygous |
| 5 | F | 9 | 7.6 | 2 | + | + | + | + | - | c.3526/3535del10 heterozygous |
| 6 | F | 6 | 4.1 | 0 | - | - | + | + | + | c.2413>T/6996delT heterozygous |
| 7 | F | 6 | 4.4 | 1 | - | - | + | - | - | c.3894/3895insT heterozygous |
| 8 | M | 12 | 8 | 2 | + | + | + | + | - | c.521-541del19 /4909+1G>T heterozygous |
| 9 | M | 11 | 8.4 | 2 | - | + | + | + | - | c.3894/3895insT heterozygous |

+, presence of specific clinical symptom; -, absence of specific clinical symptom; NA not available. Cerebellar atrophy score according to Broccoletti T et al 2011.

**Efficacy**

All patients were considered compliant to the treatment. An improvement in the total SARA score was globally evident in 5 patients during the overall treatment period. At T1, none of the subjects showed a clinically relevant effect. At T2 (0.005 mg/kg/day), an improvement of the total SARA scale from baseline was observed in 4 of the treated patients, while at T3 (0.01 mg/kg/day), an additional patient showed an improvement (Fig. 5). In detail, 3 patients exhibited a 3 points improvement, while 2 were partially responders. Two patients were not responder at all during the overall period treatment and in 2 further patients a worsening of the score was noted. Among these last 2 patients, P7, age 6 years, was the brother of P9, who, by contrast, exhibited a clear improvement even at the lowest steroid dosage. Unexpectedly, the baseline SARA score in this two subjects was 11 in P7 and 16 in P9. Moreover, P7 had a cerebellar atrophy score lower than the brother (1 *versus* 2 of the oldest patient). No difference in the basal laboratory parameters could be detected. However, even though compliant to the treatment, no reduction in ACTH plasma levels was observed during the treatment. An additional patient, P8, also exhibited a worsening of 2 points at the highest dosage. However, the worsening was exclusively limited to speech disturbance and, of note, there was for this parameter a high interrater variability. In the 5 cases, in whom an improvement was observed considering the SARA score on the whole, neurological functions returned to the baseline values during the wash-out period in 2 patients, while in the remaining the total score remained slightly lower than T0 (P1, P3 and P4), as shown in Fig. 2. A correlation between the serum ACTH levels and the clinical response was observed during the treatment ($P = 0.003$, $r = 0.62$).

Since SARA scale allows to assess 4 different domains, in order to better identify the clinical parameters which more frequently improved during betamethasone treatment, the eight items of SARA scale were grouped in the following categories: category A (gait), which includes parameters evaluating gait disturbance (gait, stance and heel-shine slide items), category B (ataxia), which includes the parameter that evaluates truncal ataxia (sitting), category C (speech), for speech disturbance and dysarthria, category D (limb ataxia), which includes

parameters that evaluate upper extremities ataxia (finger chase, nose-finger test and fast alternating movement). As indicated in Fig. 6, an improvement in gait disturbance variables and in the truncal ataxia was observed in 7/9 and 3/9 patients, respectively. Speech and limb ataxia improved only in 2 subjects. All the items which improved showed a drop of 1 point in the SARA scale. No correlation with atrophy cerebellar score was found with the age at onset, age at examination and disease duration.

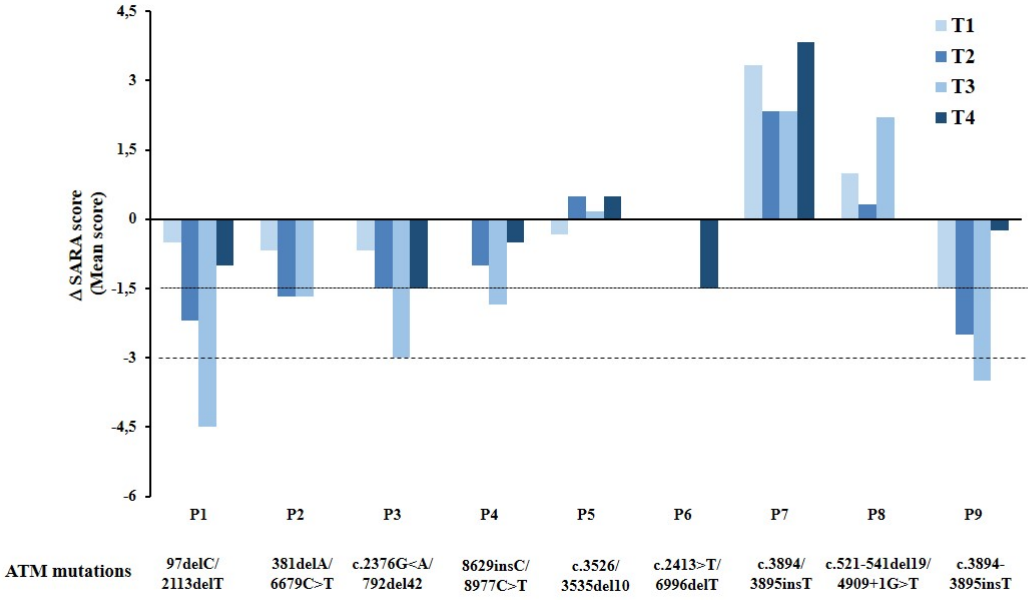


**Figure 5.** Change in SARA score during betamethasone treatment. Data are represented as mean SARA score change (negative values show improvement, zero or positive values show no change or deterioration of cerebellar ataxia) for 9 patients. No correlation between the response to the treatment and ATM mutations can be observed.

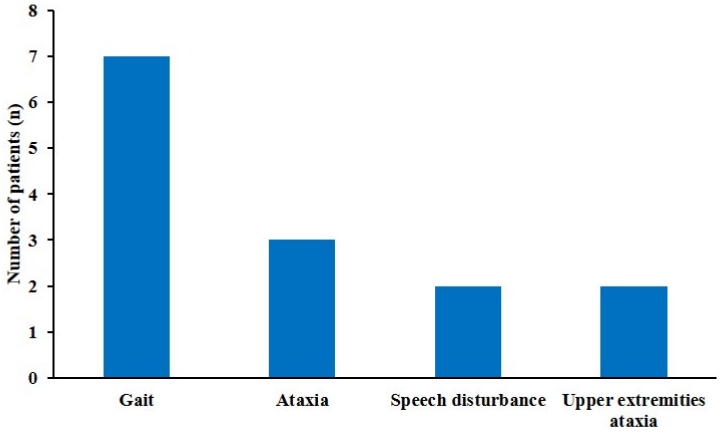


**Figure 6.** Symptoms were divided in 4 groups. Category A: gait disturbance, including gait, stance and heel-shine slide items; category B: pure truncal ataxia (sitting); category C: speech disturbance (speech); category D: upper extremity ataxia, including finger chase, finger-nose test, and fast alternating movement. Data are expressed as number of subjects showing the improvement.

**Table 2** Number of patients with a neurological improvement and entity of the Δ SARA score at each time-point of the most informative variables

| | T60 | Mean Δ SARA | T120 | Mean Δ SARA | T180 | Mean Δ SARA |
|---|---|---|---|---|---|---|
| Gait | 2 | -1 | 1 | -1 | 1 | -1 |
| Stance | 1 | -1 | 0 | NA | 1 | -1 |
| Hell shine slide | 1 | -1 | 3 | -1 | 2 | -1 |
| Sitting | 1 | -1 | 2 | -1 | 2 | -1 |
| Speech disturbance | 1 | -1 | 1 | -1 | 1 | -1 |
| Finger chase | 0 | NA | 1 | -1 | 1 | -1 |
| Nose finger | 0 | NA | 0 | NA | 0 | NA |
| Fast alternating movement | 0 | NA | 0 | NA | 1 | -1 |

NA, not applicable

**Safety and tolerability**

Only mild or moderate adverse events (AEs) occurred during the treatment period. The most common AEs were weight gain (n=8 at T3) and hypertension (n = 2 at T3) which were observed more frequently at the maximum dosage (T3) (Table 3). One patient (P2) discontinued the treatment (+15 days of the maximal dose) due to moderate weight gain without any further adverse event. Since the patient had been treated with the 0.01 mg/kg dosage for almost the 25% of the scheduled phase, the subject was not excluded from the efficacy analysis. Hypertension occurred in 2 subjects, but none of them required anti-hypertensive treatment.

Three patients experienced infections of upper airway (pharyngitis and otitis) treated with antibiotic therapy. 1 patients (P4) who suffered of bronchiectasis, chronic P. aeruginosa colonization and recurrent lower respiratory infections before betamethasone treatment, needed 3 cycles of antibiotic therapy during the 8-month trial for bronchopneumopathy exacerbation. It must be noted that in the previous 12 months this patient experienced 3 episodes of pneumonia, in one case requiring admission.

There was no notable change from baseline in the vital signs. None of the patients had neutropenia/anemia or significant worsening of lymphopenia.

No worrying pattern in clinical chemistry was observed. As for the metabolic disorders, none of the subjects had hyperglycemia/glycosuria or increased hemoglobin A1C levels, 2 patients had a worsening of pre-existing hypercholesterolemia and in 1 case a mild hypercholesterolemia could be detected at T2 and T3. In 4 and 5 patients, serum triglyceride levels increased at T2 and T3, respectively. At each time-point no behavioral difficulties or psychiatric problems related to the long-term use of glucocorticoid were reported.

**Table 3. Adverse events, according to betamethasone dosage**

| | Betamethasone | | |
|---|---|---|---|
| | T1<br>n=9 | T2<br>n = 9 | T3<br>n = 9 |
| Admission due to severe infections | 0 | 0 | 0 |
| Severe bacterial infections | 0 | 0 | 0 |
| Need of antibiotic therapy | 2 (22%) | 2 (22%) | 2 (22%) |
| Mild hypertension | 0 | 1 (11%) | 2 (25%) |
| Severe hypertension | 0 | 0 | 0 |
| Weight gain | 6 (66%) | 6 (66%) | 8 (89%) |
| Mean (SD), kg | 1.1 (1.9) | 2.25 (2.4) | 3 (2.5) |
| Median, kg | 1 | 2 | 2.8 |
| Diabetes mellitus/hyperglicemia | 0 | 0 | 0 |
| Cataract | 0 | 0 | 0 |
| Glaucoma | 0 | 0 | 0 |
| Acne | 0 | 0 | 0 |
| Myopathy | 0 | 0 | 0 |
| Psychosis | 0 | 0 | 0 |
| Increase of cholesterol levels | 0 | 3 (33%) | 4 (44%) |
| Increase of triglyceride levels | 1 (11%) | 4 (44%) | 5 (55%) |

At leastone TEAE was reported by 48% of placebo-treated and 62.8% of rotigotine-treated patients. Drug-related TEAEs were reported by 26.4% and 44.4% of placebo- an

**Change in quality of life**

CHAQ-DI was moderate-severe in 7/9 patients at T0 (mean 2.18+/-1.1, range 2.11-2.47), while in 2 patients it was mild (P3, 0.12) or mild-moderate (P6, 0.62).

In detail, the most affected categories were those concerning dressing, hygiene, reaching and activities. During the treatment period, the CHAQ-DI on the whole did not change significantly in 8/9 patients, with the exception of P7, who despite the worsening in SARA scale, had a reduction of approximately 23 and 18% in CHAQ-DI at T1 and T2, respectively. In a further subject (P4) the CHAQ-DI decreased, even though the change did not reach the cut-off of 15% (13%).

When each of the 8 categories was analyzed individually, an improvement was noted in 4/9 patients at T2 in hygiene tasks, while in 3 patients an improvement was noted in dressing, grip and reaching. In the analysis of the 30 items of individual

subjects, we noted that 4 patients (P1, P7, P8, P9) at T2 exhibited an improvement ≥ 5 specific daily life activities (DLA). However, the improvement of individual tasks didn't affect the overall CHAQ-DI, since most of the 30 items didn't change. It should be noted that the overall impact on the QoL was variable in that a worsening was observed for 5 categories in P9, who, however, had an improvement at the SARA score. Three further patients had a mild worsening of the reaching category.

**Discussion**

A-T remains at the moment an incurable disease with a very short life span and a poor QoL. Unlike other forms of PIDs, definitive therapies based on innovative approaches, as gene therapy or gene editing technologies, are still far from being available. In this perspective, the possibility of using disease modifying agents, able to attenuate the disabilities, although temporarily, and improve QoL is widely desirable.

In the last 10 years a number of drugs has been under investigation for the symptomatic treatment of A-T. In ATM deficient mice, the effect of some antioxidants, such as N-acetyl-L-cysteine, EUK-189, tempol and 5-carboxy-1,1,3,3-tetramethyl isoindolin-2-yloxyl, has been tested for their chemo-preventive properties (57). Glutamine supplementation is able to rescue the decrease in brain-derived neurotrophic factor expression and the nuclear translocation of histone deacetylase 4, resulting in improved health and life span (58). Furthermore, the biological role of several molecules, including aminoglycoside antibiotics and antisense morpholino-oligonucleotides, has been tested in vitro. These molecules could potentiality restore ATM functions, even though their translation into the clinical setting is still far from being achieved due to their safety/side effect profiles (REF).

Glucocorticoids are currently the only medication that has been shown to benefit neurological A-T phenotype. In particular, in previous studies, speech disturbance and stance, as well as the quality of motor coordination, were the more sensitive neurological parameters (53, 54). More recently, in a phase 2 study, it was observed that infusions of autologous erythrocytes loaded with dexamethasone

(EryDex) were effective in improving neurologic symptoms in a few A-T patients. However, the procedure is quite invasive since it requires the collection of 50 ml of peripheral blood from the patients and the subsequent intravenous re-infusion (59). Increased activation in relevant cortical areas has also been documented in A-T patients, who exhibited a good motor response to betamethasone treatment, thus suggesting that GCs could facilitate cortical compensatory mechanisms (60). However, despite these observations, up to date no data are available on the minimal effective dosage of the drug. This is important, since GCs treatment is associated to several side effects.

In this trial, we documented that 4 out 9 patients have a benefit at the dose of 0.005 mg/kg per day of oral betamethasone. Using the higher dosage, only 1 additional patient had a positive response. Conversely, a daily dose of 0.001 mg/kg was ineffective. Gait disturbance and truncal ataxia variables were the most sensible parameters.

The molecular basis to explain the benefit of betamethasone on A-T is not well defined. On the other hand, the intimate mechanism of neurodegeneration in A-T is still poorly defined (61). ATM is predominantly a nuclear protein, however a number of studies reported that it is also present in the cytoplasm, within cytoplasmic vesicles, peroxisomes and mitochondria, where it plays additional functions independent of its role in the DNA damage response (17). Abnormalities in cell-clearance processes characterized by an inappropriate fusion between autophagosome and lysosomes have been recently reported in A-T cells. It has been found that betamethasone can interfere in the process by exerting an *in vitro* positive effect on molecules implicated in autophagosome degradation (62).

Furthermore, a non-canonical splicing event in the ATM mRNA precursor, referred as ATMdexa1, has been demonstrated in lymphoblastoid cell lines derived from A-T patients *in vitro* cultured in the presence of dexamethasone or in some patients treated with EryDex **(**63). The expression of these transcripts was drug-dependent and well correlated with the patients' responsiveness to the therapy, suggesting the possibility to use these molecules as prognostic marker (64). Again, several differentially expressed genes, implicated in the biochemical involving ATM, are restored by dexamethasone and are currently under investigation (65).

In addition to its activation by double stand breaks, ATM has been found to be activated in the cytoplasm by ROS. Oxidative stress is considered to have a crucial role in the A-T physiopathology. Some evidence suggests that GCs may act in redox homeostasis, by potentiating antioxidant defenses through an increase of the antioxidants molecules, glutathione (GSH) and NADPH. Moreover, GCs also promote the nuclear accumulation of the transcription factor nuclear factor (erythroid-derived2)-like 2 involved in GSH and NADPH pathways. Of note, a remarkable reduction in ROS levels was documented in response to betamethasone treatment (55, 66).

Taken together these considerations led to hypothesize that the effects of GCs are far beyond the anti-inflammatory properties of the drug, potentially interfering in the intimate pathogenic mechanisms.

Thus far, all the studies conducted on GC therapies in A-T have documented a variability in the clinical response, in that a few patients clearly improved and a few do not at all. Our study confirms this variability. This response does not depend on the residual amount of expression of ATM itself, thus implying that interfering factors, biological, molecular, envinromental or drug-related, are powerful modifiers of the neurological phenotype. Due to this variability in the response to the treatment, there is a need for biomarkers to predict the response to GCs at the beginning of the treatment. We observed a correlation between the reduction of ACTH serum levels during GC treatment and the ΔSARA score, thus suggesting that this parameter may help clinicians in predicting the clinical response.

This study points to dosing modulation to harness the beneficial effects of GCs on neurological phenotype, avoiding the deleterious consequences of high dosage therapy. Overall, the AEs observed in this study were moderate, being limited to weight gain and mild hypertension. Only 1 patient discontinued the treatment after two weeks at 0.01 mg/kg/day because of weight gain. The major expected risk with betamethasone in such patients was a significant increase of the infectious risk. However, no severe/recurrent infections occurred during this study, and the frequent exacerbations of lung infections in the patient P4 were more likely due to the pre-existent lung condition rather than to an immunosuppressive effect of GC therapy. No behavioral or other severe side effects were observed during the 6 month-therapy.

As for QoL we did not observe improvement in the CHAQ-DI, on the whole, in the majority of the patients. This may be due to the fact that most of the patients had an advanced form of the disease, which may have influenced both parents and patients' perception of the condition. Furthermore, since the index is strongly influenced by the worst performance in the individual items, a significant improvement in the index was not possible despite the improvement of some DLA. However, it is noteworthy that 5 items improved in 4 patients. This finding should be taken into consideration in evaluating the effect of the GC treatment on the QoL.

In conclusion, our findings would suggest that an occasional usage of short-term betamethasone oral treatment, at a daily dosage of 0.005 mg/kg, could be allowed under the medical supervision. Pre-existing risk factors for adverse side-effects should be taken into account before the start of the treatment.

**Supplementary Table 1**

Intraclass inter-evaluator correlation coefficient during the trial

| Item | Inter-rater reliability (ICC)(n-3) |
|---|---|
| Gait | 0.95 |
| Sitting | 0.60 |
| Hell-shine | 0.66 |
| Stance | 0.91 |
| Finger chase | 0.66 |
| Nose finger | 0.85 |
| Fast alterning movement | 0.42 |
| Speech disturbance | 0.90 |
| Total Δ SARA score | 0.77 |
| Total SARA score | 0.89 |

To assess the reliability of SARA score we also evaluated intraclass inter-evaluator correlation coefficient (ICC). Single items had a good (between 0.60-0.74) or excellent (between 0.75-1.00) inter-rater reliability with ICCs. Only for fast alternating movement item ICC was fair (between 0.40 and 0.59)

## Conclusions

Differently from other forms of PIDs whose clinical phenotype is predominated by the increased susceptibility to infections, A-T is the prototype of more complex syndromes in which the immunodeficiency is only one of the multiple components of the disease. The clinical phenotype is mainly characterized by progressive neurodegenerative process, especially affecting the cerebellum. It is to note that, unlike lymphocytes, whose turnover is continuous, Purkinje cells are mature and differentiated cells which are not subject to turnover. The QoL in A-T patients is dramatically affected by the neurological impairment, which almost invariably confines these patients to wheelchair by the age of ten years.

Unfortunately, currently there is no effective treatment to cure or prevent the progress of neurological deterioration in A-T, but only supportive care. As for the intimate molecular mechanism by which betamethasone led to this effect it is not possible to give a definitive interpretation, given that the pathogenesis of neurodegeneration itself is still far from being clear.

Thus, the identification of the potential site of action of steroids in A-T will open a new window of intervention in this so far non-curable disease. Eventually, the identification of a pathogenic role of abnormal autophagy-lysosomal pathway in A-T will be extremely useful in indentifying innovative therapeutic strategies and new drug targets.

The data herein reported are under review to the *European Journal of Neurology* as original article.

# CHAPTER II

## "Characterization of Patients With Increased IgM Levels, B-Cell Differentiation Blockage, Lymphoproliferation and DNA Repair Defect "

The earliest evidence that individuals with PIDs develop cancer was reported in 1963 (67). An increasing number of reports subsequently indicated that subjects with congenital abnormalities of the immune system are at a high risk for developing cancer including lymphoma and stomach, breast, bladder, and cervical epithelial cancers (67).

The overall risk for children with PIDs of developing malignancy is estimated at 4–25% (68, 69). The type of malignancy that is seen is highly dependent on the precise PID, the age of the patient, and probably viral infection indicating that different pathogenic mechanisms may be implicated in each case (68). According to the Immunodeficiency Cancer Registry (ICR) database NHL and Hodgkin's disease (HD) account for 48.6 and 10%, respectively, of the malignancies seen in patients with PIDs. Genomic instability due to defective DNA repair processes and other unknown mechanisms in PID patients leads to an enhanced risk of cancer.

The findings of elevated serum IgM with low IgG, IgA, or IgE in the setting of immune deficiency leads most immunologists toward a diagnosis of Hyper IgM syndrome (HIGM), rare inherited PIDs characterized by class switch recombination defects (CSR) and sometimes impaired somatic hypermutation (SHM). SHM plays a role in the selection and proliferation of B cells expressing a B-cell receptor (BCR) with a high affinity for the antigen and does require the integrity of the cell DNA repair machinery.

The majority of these forms are caused by X-linked (XL) or autosomal recessive (AR) defects in the CD40 ligand/CD40 signaling pathway or AR disorders involving activation-induced cytidine deaminase (AID), or in the uracil DNA glycosylase (UNG). Other HIGM are caused by mutations or in the X-linked nuclear factor k-B essential modulator *(*NEMO*)* gene*,* reported in patients affected with ectodermal dysplasia. In all these cases, both SHM and CSR processes are equally impaired. The unique condition, so far described, of dissociation between the CSR and SHM process is represented by the autosomal dominant mutation in C terminal end of AID in patients showing defective CSR but intact SHM. This observation would imply a different molecular control of the 2 processes. To date, in spite of the identification of new genetic defects associated with HIGM through NGS

technologies, in about the 15% of the cases of HIGM patients, the molecular defect still remains to be defined.

The clinical phenotype of HIGM is invariably severe and characterized by increased susceptibility to recurrent bacterial and opportunistic infections, neutropenia, autoimmunity and cancer susceptibility.

The presence of elevated levels of IgM were also reported associated with other immunological defects like RAG2, ATM and ARTEMIS deficiency or to acquired causes, such as in autoimmune diseases, with IgM autoantibodies, or in chronic infections. In B-cell lymphoproliferative disorders, elevated monoclonal IgM levels may also be found.

In this paper, submitted on *Frontiers in Immunology*, we report on a group of unrelated patients with very high polyclonal IgM levels, resembling a HIGM of unknown molecular defect, whose clinical course was complicated by the occurrence of a lymphoproliferative disorder. In these patients an evaluation of B-cell subsets has also been performed, revealing a reduction of memory and switched memory B cells. Through the comet alkaline and micronucleus (MN) assays on peripheral blood lymphocyte or fibroblast cultures an increased genotoxicity was documented. In order to evaluated a molecular cause of the disorder NGS analysis was performed, revealing in two subjects mutations in PIK3R1 or ITPKB genes, implicated in B- and T-cell development..

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# Elevated IgM levels with defect in somatic hypermutation and increased susceptibility to lymphoproliferation

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**The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest**

### Author contribution statement

V.G., E.C., C.P. organized, collected and analyzed the data; V.G., C.P. wrote the manuscript; G.G., R.P., A.S., L.d.V., G.S., A.D., M.R.S., performed the experiments; V.Ma., V.Mo., contributed with samples from some patients; G.DM., C.S., performed whole exome sequencing; all authors reviewed and approved the manuscript.

### Keywords

hyper-IgM syndrome, Lymphoproliferative Disorders, Class switch recombination, somatic hypermutation, DNA Damage, DNA Repair



### Abstract

Word count: 199

Elevated IgM levels represent a hallmark of Hyper IgM syndromes, rare inherited immunodeficiencies characterized by a defect of class switch recombination, sometimes associated to impaired somatic hypermutation. DNA repair defects or common variable immunodeficiency may also associate with a hyper-IgM phenotype. We report on 7 patients showing high polyclonal IgM levels, impaired B-cell homeostasis with reduced memory and switched-memory B cells, and a high incidence of lymphoproliferative disorders. Through micronucleus assay, an increased genomic instability was documented in 2 of the patients studied, who developed a lymphoproliferation. Next generation sequencing revealed in two of them mutations in the PIK3R1 or ITPKB genes, implicated in B- and T-cell development, survival and activity. Recently, heterozygous gain of function mutations in the PIK3R1 gene were reported in a novel form of combined immunodeficiency and elevated IgM with reduced class-switched memory B cells. While a deletion of a region containing the ITPKB and a CVID phenotype has been already documented, this is the first report of a point mutation of this gene. This report gives further insight into the B-lymphocyte disorders and highlights a possible role of polyclonal hyper-IgM as a biomarker of immune dysregulation, particularly affecting B-cell homeostasis, and susceptibility to lymphoproliferation.

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### Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

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The study was carried out in accordance with the Declaration of Helsinki and was approved by the institutional ethics committee Federico II. Informed consent was obtained from all patients before the study.

**Original article**


## Abstract

Elevated IgM levels represent a hallmark of Hyper IgM syndromes, rare inherited immunodeficiencies characterized by a defect of class switch recombination, sometimes associated to impaired somatic hypermutation. DNA repair defects or common variable immunodeficiency may also associate with a hyper-IgM phenotype. We report on 7 patients showing high polyclonal IgM levels, impaired B-cell homeostasis with reduced memory and switched-memory B cells, and a high incidence of lymphoproliferative disorders. Through micronucleus assay, an increased genomic instability was documented in 2 of the patients studied, who developed a lymphoproliferation. Next generation sequencing revealed in two of them mutations in the *PIK3R1* or *ITPKB* genes, implicated in B- and T-cell development, survival and activity. Recently, heterozygous gain of function mutations in the *PIK3R1* gene were reported in a novel form of combined immunodeficiency and elevated IgM with reduced class-switched memory B cells. While a deletion of a region containing the *ITPKB* and a CVID phenotype has been already documented, this is the first report of a point mutation of this gene. This report gives further insight into the B-lymphocyte disorders and highlights a possible role of polyclonal hyper-IgM as a biomarker of immune dysregulation, particularly affecting B-cell homeostasis, and susceptibility to lymphoproliferation.

## Introduction

Immunoglobulin M (IgM) represents the first line antibody to be produced during an immune response, that follows antigen encounter. The presence of high IgM levels may be due to rare immunological disorders, such as hyper IgM syndromes (HIGM), but also to autoimmune diseases or acquired infectious causes (1, 2). In B-cell lymphoproliferative disorders, elevated IgM levels may be found, but they are monoclonal.

HIGM are characterized by normal or increased IgM levels and absent or strongly reduced levels of the other Ig isotypes due to impaired class switch recombination (CSR), which sometimes parallels an abnormal somatic hypermutation (SHM). Both processes require the integrity of the cell DNA repair machinery, even though different repair mechanisms are implicated. Within the genetic disorders, 6 genes have been so far implicated, coding for molecules involved in the CD40/CD40 ligand signaling *(CD40L, CD40)* (3, 4) or in cytosine/cytidine deaminase process *(AID)* (5). Other HIGM are caused by mutations in the uracil DNA glycosylase (*UNG*)(6) or in the X-linked nuclear factor k-B essential modulator *(NEMO)* genes, reported in patients affected with ectodermal dysplasia, and finally in patients with post-meiotic segregation 2 (*PMS2*) deficiency (7, 8). Overall, the clinical phenotype of the inherited forms of such disorder is variably severe and characterized by increased susceptibility to recurrent bacterial and opportunistic infections associated with autoimmunity and cancer susceptibility. Elevated IgM levels were also reported associated with other immunological defects, causing well-defined immunodeficiencies as part of a heterogeneous clinical phenotype, like *RAG2*, *LRBA*, *ATM* and *ARTEMIS* deficiency (9-12). However, in spite of the introduction into the clinical setting of next generation sequencing (NGS) technologies, which allow a rapid identification of already known genetic variations or novel defects, a few immunodeficiency conditions,

including those with elevated IgM levels, still remain to be elucidated in the intimate molecular alteration (1).

We report on 7 unrelated patients with high polyclonal IgM levels, whose clinical course was complicated in 5 cases by the occurrence during the follow-up of a monoclonal lymphoproliferative disorder (LPD). The evaluation of B-cell subsets revealed inconstantly a reduction of memory and switched memory B cells. An increased genomic instability was documented, through the classical micronucleus assay in lymphocytes from 2 out of the 4 tested patients. Next generation sequencing revealed in two patients mutations in the phosphoinositide-3-kinase regulatory subunit 1 (*PIK3R1),* and inositol 1,3,4, trisphosphate kinase β *(ITPKB)* genes, both of them playing a role in promoting B- and T-cell development, survival and activity.

In this study, we document a novel immunodeficiency condition, characterized by impaired B-cell homeostasis and increased susceptibility to genotoxic agents and to cancer development.



## Patients and Methods

In 7 patients (four female) from unrelated non-consanguineous families, high IgM levels (range 443-911 mg/dl) were documented and were enrolled in the study. The study was carried out in accordance with the Declaration of Helsinki and was approved by the institutional ethics committee. Informed consent was obtained from all patients before the study. Peripheral blood samples were collected in heparin or ethylenediamine tetraacetic acid (EDTA) and processed within 24 h. Genomic DNA was isolated from peripheral blood lymphocytes using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

**B-cell immunophenotyping and CD40L expression**

Whole blood anti-coagulated with EDTA was used for multi-color flow cytometry immunophenotyping and processed within 24 h. Peripheral blood mononuclear cells (PBMCs) were obtained by density-gradient centrifugation using standard procedures. Cells were exposed to directly conjugated mouse anti-human monoclonal antibodies to assess lymphocytes with multi-color flow cytometry immunophenotyping using the following fluorochrome conjugated antibodies: anti-CD45 peridinin chlorophyll (PerCP) (clone TU116), anti-IgD phycoerythrin (PE) (IA6-2; all from BD Biosciences, San Jose, CA, USA), anti-CD19 allophycocyanin (APC) (clone SJ25-C1), anti-CD24 fluorescein isothiocyanate (FITC) (clone SN3), anti-CD38 phycoerythrin (PE) (clone HIT2), anti-CD27 FITC (M-T27; all from Invitrogen/Caltag, Karlsruhe, Germany) and anti-CD21 fluorescein isothiocyanate (FITC) (clone 1F8, Dako, Glostrup, Denmark). The cells were incubated with directly labelled antibody at 4°C in the dark for 30 min, washed and re-suspended in 100 ml of PBS. Flow cytometric analysis was performed on a BD FACS Canto II flow cytometer (BD Biosciences) and analytical flow cytometry was performed using BD FACSDiva software. The antibody combinations used were: (1) anti-CD27 FITC, anti-IgD PE, anti-CD45 PerCP, anti-CD19 APC; (2) anti-CD24-FITC, anti-CD38 PE, anti-CD45 PerCP, anti- CD19 APC; and (3) anti-CD21 FITC, anti-CD38 PE, anti- CD45 PerCP, anti-CD19 APC. The events in the displayed graphs were gated by forward (FSC) and side scatter (SSC) to exclude dead cells. Lymphocytes were identified by gating on viable $CD45^+$ cells and B-lymphocytes were gated on $CD19^+$ cells. CD40L and CD40 staining were performed through directly conjugated mouse anti-human monoclonal antibodies.

**Study of class switch recombination and somatic hypermutation *in vitro***

PBMC were cultured in the presence of 500 ng/ml of soluble CD40-L and 100 U/ml of rIL-4. Proliferation was assessed by measuring [3H] thymidine uptake. On day 5, total RNA from activated cells was extracted with Trizol (Invitrogen), and converted in cDNA by the use of reverse transcriptase. The presence of Iε-CHεgermline and functional $V_H$-Cγ1 and $V_H$-Cε transcripts was assessed by RT-PCR as previously described (13). IgE production was evaluated in supernatants by ELISA on day 12 (14).

SHM generation in the VH3-23 region of IgM on purified CD19+CD27+ B cells was also performed in two patients as previously described in detail (5). PBMCs were isolated by flow cytometry using FITC-anti-CD19 and PE-anti-CD27 monoclonal antibodies (Immunotech). RNA was purified with the Trizol reagent and cDNA was obtained by reverse transcription with an oligo dT primer. PCR was carried out with the Pfu polymerase (PfuTurbo, Stratagene) using primers for the VH3-23 leader exon (5V GGCTGAGCTGGCTTTTTCTTGTGG-3V) and CA region (CAB; 5V-TCACAGGAGACGAGGGGGAA-3V) (35 cycles at 94-C for 45 s, 60-C for 1.5 min, 72-C for 2 min). PCR products were subcloned and analyzed as described above.



**Comet and micronuclei assays**

In 4 patients (P1, P2, P4, P5,) and 4 healthy age-matched donors, the cytokinesis-block micronucleus (MN) assay and the alkaline comet assay were performed to test spontaneous or chemically induced DNA damage and DNA migration, respectively. For each donor, 4 lymphocyte cultures were set up following standard methods to evaluate spontaneous and Mitomycin-C-induced MN frequency. Cytochalasin-B was added 44 h after phytohaemagglutinin (PHA) stimulation. After further 72 h of growth, cultures were harvested and slides were made-up. For each sample, 1000 cytokinesis-blocked cells were examined

to record MN (15). The spontaneous and Methyl-metane-sulphonate-induced percentage of DNA migration, alkaline comet assay, were analyzed on 200 randomly selected cells for each sample (16).

### Whole exome and Sanger sequencing

Whole exome sequencing (WES) was performed in 6 cases, while direct Sanger sequencing of the *CD40LG, CD40*, *AICDA*, *UNG*, *NEMO*, *TNFRSF13B*, *ATM*, *PIK3CD, PIK3R1* genes, was performed in selected cases. Samples were sequenced to at least 2.5 GB on an Illumina MiSeq with TruSeq MiSeq V3 reagents, yielding paired 250 nucleotide reads. Samples were prepared for exome sequencing using the TruSeq HT library preparation kit (Illumina; San Diego, CA, USA) and subsequent exome enrichment through the xGen Exome Research Panel V1.0 (Integrated DNA Technologies; Coralville, IA, USA) according to manufacturers' protocols. Alignment, variant calling, and analysis were carried out, as previously described (17, 18).



## Results

### Clinical and immunological data of HIGM-like patients

As shown in Table 1, 6 of the seven patients studied suffered from recurrent bacterial and viral infections, in particular of the upper and lower respiratory tract, causing in P1 a significant lung damage characterized by pulmonary lower lobes bronchiectasis and lobar middle atelectasis detected at chest high resolution computed tomography. Atelectasis were also found in P5, whereas interstitial lung disease was documented in P2. P1 and P2 also experienced a measles complicated by pneumonia (P1) and a chickenpox with severe ocular involvement (P2). Quantitative PCR for EBV, CMV, HCV-RNA, and HBV-DNA, was

negative in all patients with hypogammaglobulinemia, who underwent Ig replacement therapy. Involvement of the reticulo-endothelial system with lymphadenopathy, liver and spleen enlargement was documented in 6 patients. Four patients had autoimmunity or signs of chronic inflammation: hemolytic anemia, immune thrombocytopenia, autoimmune myofascitis and recurrent fever. Other clinical features included urticarial-like lesions or other recurrent skin lesions in 5 cases, growth retardation and delayed puberty in 2, mood and behavioral disorders in 2 and Arnold-Chiari malformation in one further patient. In five cases, IgG levels were 2 SD below mean value of age-matched controls, whereas in the remaining cases, IgG were normal (range: 858-1500 mg/dl); IgA isotypes were normal in all cases (range: 33-282 mg/dl), except for P6 and P7, who showed a total IgA deficiency.

Five patients of our case series developed during the follow-up a B-cell LPD, as detailed in the Table 1. P1 received a diagnosis of non-Hodgkin marginal zone B-cell lymphoma (NHL) with a cutaneous infiltrate at age of 11 y, thereafter requiring matched related donor haematopoietic stem cell transplantation (HSCT). P2 was diagnosed at 47 y with a low-grade mucosa-associated lymphoid tissue (MALT) lymphoma. Helicobacter pylori (HP) infection was also detected and when eradicated the remission of MALT was observed. P3 received a diagnosis of high-risk acute lymphoblastic leukemia at age of 3 y, requiring at first HSCT from matched unrelated donor, and thereafter anti-CD20 mAb therapy due to an EBV-related LPD. P6 received successful chemotherapy for a cervical Hodgkin lymphoma at 19 y. P7 at 15 y of age was treated for a diffuse large B-cell lymphoma, invading the colonic mucosa, but the patient died from infection, as already reported (19). In 2 patients (P1 and P2) it was possible a long-term longitudinal evaluation of IgM levels well before the onset of the LPD, as illustrated in Figure 1. Both patients had elevated IgM levels, ranging from 680 and 750 mg/dl in P1 and from 910 and 1060 mg/dl in P2. In both patients no

significant further increase in IgM levels was observed when they were diagnosed as LPD, while a significant reduction was documented in P1 in the subsequent 4 years post-chemotherapy (range 280-386 mg/dl). In P3, hypogammaglobulinemia was first noted. Thereafter, a progressive increase of IgM levels occurred after a HSCT, performed for an acute lymphoblastic leukemia. P3, at the age of 9 years also developed autoimmune thrombocytopenia and 1 year later required prednisone treatment for the onset of an autoimmune hemolytic anemia. Bone marrow aspiration showed normal cellularity. In both P6 and P7, elevated IgM levels were documented in the first years of life during an immunological evaluation required for bacterial recurrent infections, and persisted high at the time of the LPD onset.

In P6 and P7, HIGM-like phenotype was associated with a profound T-cell defect at the time of the LPD diagnosis, characterized by a progressive lymphopenia, predominantly involving the CD4 naïve compartment in patient 7, and decreased T-cell proliferation in both patients. In the remaining patients, no significant abnormalities were found in the T-cell compartment (**Table 2**).



**B-lymphocyte profiling**

B-lymphocyte phenotyping revealed that the percentage of total CD19+ B lymphocytes was reduced in P2, P4, P6 and P7 (5, 2, 4 and 0%, respectively) and normal in the remaining patients, as compared to age-matched reference values (20). CD19+CD20-IgG+ mature B cells were absent in all patients. Circulating CD19+CD27-IgD+ B cells (naive B cells) were normal or low/normal in all patients investigated (P1-P5), implying a normal central B-cell development. The percentage of CD19+CD27+IgM+ memory B cells were significantly reduced in 4 out of the 5 patients (range 1.6-5% of CD19+cells) (P1, 3, 4, 5) and only slightly

reduced in P2, as compared to reference values (21). A significant reduction of CD19+CD27+IgM- switched memory B cells was also observed in 3 of the 5 patients studied (range 0-4.6% of CD19+cells) (P1, 2, 3) (**Table 2**). Intriguingly, as shown in Figure 2, all the patients (P1, P2 and P3), who developed lymphoproliferative disorders, in whom it was possible to perform the extended B-cell phenotyping, showed a significant alteration in both B-cell subsets, and predominantly in the switched memory population. However, these patients exhibited a normal capability to produce specific IgG antibodies. Unfortunately, these data were not available for P6 and P7, who also developed LPD-disorders.

CD40L and CD40 expression was normal in all cases except for P4, in which CD40L expression was reduced, albeit *CD40LG* gene alterations were ruled out by direct sequencing.



**Reduced SHM with normal *in vivo* and *in vitro* CSR**

Besides the increased IgM serum levels, all patients had IgG specific antibodies as shown in Table 3, indicating a normal *in vivo* CSR. This finding was also observed in some hypogammaglobulinemic patients before the start of replacement therapy. In 2 of them (P1 and P2), the activation of B cells through sCD40L+IL4 confirmed *in vitro* a normal CSR, at least towards IgE (**Table 3**). In addition, in these patients a normal B-cell proliferation following the exposure to IL4+sCD40L was also found (data not shown).

The study of SHM showed a polyclonal distribution in all the 4 patients evaluated (P1, 2, 4, 5). In the only 2 cases (P1 and P2), in whom it was possible to evaluate the mutation rate, it was found significantly reduced (1.9 and 2.3% *vs* 3.5-6.3% of controls), although the nucleotide substitution pattern was normal.

**Increased genomic instability at micronuclei assays**

Since elevated IgM levels and a high susceptibility to lymphoproliferative disorders have been reported in Ataxia-teleangiectasia, a prototype of DNA repair machinery defects, an underlying DNA instability and repair deficiency was hypothesized, also on the basis of the reduced SHM documented in P1 and P2. Thus, to evaluate the entity of the DNA damage, a MN and CA assays were performed in 4 patients and 4 healthy donors. As shown in Figure 3A, the MN assay revealed that in the 2 patients with LPD (P1 and P2) the mean value ± SD of spontaneous MN was higher than the 2 patients without LPD (P4 and P5) and controls (15.5 ± 4.9 *vs* 5.5 ± 0.7 and 7.5 ± 4.5, respectively). In the patients with LPD, the value of MN was ≥ the upper control limit of 12 MN/1000 binucleated cells (22, 23). Moreover, when the DNA damage was evaluated after chemical stress, the patients with LPD showed a higher sensitivity to suboptimal, low or medium, concentrations of MMC than patients without LPD or controls, even though the difference was not statistically significant for the low number of patients studied (**Figure 3B**). On the contrary, no difference was observed between the 3 subgroups under the maximal DNA damage stress conditions. As for CA, we found comparable results between control and patient cells, which ruled out an alteration in the induction and repair of methyl sulfonyl methane-induced DNA damage (data not shown).

**Next Generation Sequencing**

Recently, mutations in the *PIK3CD* or *PIK3R1* genes have been reported in novel forms of primary immunodeficiency, characterized by elevated IgM and normal to strongly reduced IgG serum levels, lymphopenia, respiratory infections, lymph node enlargement and elevated risk of lymphomas (24-27). Sanger sequencing of these genes resulted normal in the 5 cases studied (P1, 2, 3, 4, 5). WES revealed in P6 a *de novo* splice site mutation in

*PIK3R1* (c.1425+1G>T), already reported (26, 27), and in P4 a heterozygous frameshift mutation in *ITPKB* gene (c.146_147insA), an unreported variant, predicted to damage protein function with high confidence (**Figure 4**). Recently, a microdeletion of the chromosome 1q42.1-42.3, involving *ITPKB* gene, has been reported in a CVID patient, suggesting a potential pathogenetic role for this gene (28). In the remaining patients, heterozygous variants of unknown significance, according to the American College of Medical Genetic criteria, were identified in several genes (*CD3 zeta*, *CASP10*, *UNC13D*, *EPG5*, *NCF2*), that were not consistent with the clinical and immunological phenotype of the patients.



## Discussion

Here, we report on a peculiar phenotype characterized by very high polyclonal IgM levels, but normal CSR, associated with high incidence of B-cell tumors, alterations of memory B-cell subsets and reduced SHM. Elevated IgM levels were not directly related to LPD and preceded for a long time the onset of LPD. In spite of a few similarities with genetically determined HIGM, the immunological and molecular evaluation of our case series recalls an entity distinct from known HIGM. In order to better define this novel immunodeficiency condition, we first studied the B-cell compartment through an extended B-cell immunophenotyping. The analysis revealed an alteration of the B-cell maturation process, characterized by a reduction of memory and class-switched memory B cells, suggesting an altered germinal center function. Notably, there was a significantly more pronounced defect of the CD19+CD27+IgM- switched memory subset in patients who developed LPD. These B-cell abnormalities have also been reported in patients affected with CVID (29, 30). Of note, CVID patients are also prone to develop lymphomas (31). Indeed, a significant correlation

between high serum IgM levels at diagnosis and the eventual development of either polyclonal lymphocytic infiltration or lymphoid malignancy in CVID patients was reported (32). In addition, polyclonal hyper IgM and development of B-cell lymphoproliferative malignancy were also found in a rare disorder, namely persistent polyclonal B-cell lymphocytosis, even though this form was characterized by a persistent expansion of CD27+IgM+IgD+ memory B cells (33), differently from what we observed in our patients. However, even though a few patients along with elevated IgM also showed low IgG levels, in none of the 7 patients the CVID criteria were fulfilled (ESID diagnostic criteria). In particular, the other IgA and IgM isotypes were normal or even high and isohemagglutinins were present in all patients except for P6 and P7. A combined reduction of both unswitched and switched memory B cells was also reported in other forms of primary immunodeficiency, including the X-linked lymphoproliferative syndrome type 1, the Warts, Hypogammaglobulinemia, Infections, Myelokathexis (WHIM) syndrome, or the DOCK8 (Dedicator of cytokinesis 8) deficiency (34-36). In the latter condition, an impaired Toll-like receptor 9-Myeloid differentiation primary response gene 88 (TLR9- MYD88) signaling pathway, in which DOCK8 functions as adaptor molecule, was found underlying the defect of memory B cells and memory maintenance.

Due to the elevated IgM levels, we further investigated the CSR and SHM of IgM on CD19+CD27+ B cells. CSR and SHM represent the two major maturation events required for an efficient humoral response, and both take place simultaneously in the germinal center after CD40 activation. Intriguingly, differently from classical HIGM, we observed a normal *in vivo* and *in vitro* CSR, but a reduced frequency of SHM in the two tested patients. These findings would favor the hypothesis of an underlying altered germinal center functionality, as previously suggested by the reduced memory and class-switched memory B cells.

Unfortunately, because of chemotherapy, HSCT or death, we were not able to test more patients for SHM. The decrease of SHM, along with a normal in vitro CSR, is a rare event (37), interestingly suggestive of a DNA repair pathway defect different from Non-Homologous-End-Joining (NHEJ) or Ataxia-teleangiectasia (A-T) and Nijmegen breakage protein (NBP) syndromes, implying the involvement of mismatched repair enzymes (MMR) and error-prone polymerases pathways, which are selectively involved in SHM. At the moment, the unique condition of dissociation between CSR and SHM processes is represented by defects of AID C terminal, UNG or PMS2 genes, in which patients show defective CSR but normal frequency of SHM, even though this was not the case in our cohort of patients.

Studies on the susceptibility to DNA damage revealed that there was an increased spontaneous frequency of micronuclei only in the patients who developed a LPD. This finding was in keeping with what occurs in DNA repair defects, although at CA no significant alteration in the induction and repair of induced DNA damage was found. As matter of fact, the high incidence of LPD occurrence in this immunodeficiency condition seems to be the hallmark of this disorder, that along with the impaired SHM, reinforce the hypothesis of the presence of increased susceptibility to DNA damage related to a DNA repair deficiency. However, it should be mentioned that the predisposition of PID patients to cancer could also be due to the immunodeficiency itself, as tumor immune surveillance becomes impaired and infections by potentially oncogenic viruses are less likely to be dealt with efficacy (38). In the last years, several PID-associated DNA repair proteins have been described, mostly of them affecting one or more processes among V(D)J recombination, CSR and SHM mechanisms. Similarly to our cohort, also in patients with alterations of proteins acting downstream of AID, such as ATM and NBP, or in patients with Artemis or mismatched repair enzymes (MMR)

deficiencies, high IgM levels and increased cancer susceptibility have been reported (39, 40), indicating that several pathways are implicated in protecting the cell from genotoxic damage.

Our case series, was finally genetically analyzed firstly through direct sequencing of genes expected to be involved on the basis of clinical and immunological phenotype, and subsequently through next generation sequencing, since no genetic alteration were found using the classic diagnostic approach. WES allowed to identify a variation in 2 genes implicated in the B- and T-cell development and function, *PIK3R1* and *ITPKB*. Recently, heterozygous gain-of-function mutations in *PIK3R1*, encoding for p85α, one of the catalytic subunits of the PI3 Kinase molecules, were reported as responsible for a novel form of immunodeficiency in 12 patients (26, 27)**.** This novel immunodeficiency, similarly to activated PI3 kinase delta syndrome (APDS) due to mutations of another subunits of the PI3K pathway, p110δ, is characterized by elevated IgM and low IgG serum levels, recurrent respiratory infections, lymph node enlargement, poor growth, and elevated risk to develop lymphomas. The *ITPKB* gene variation has never been reported previously, even though a deletion involving this gene has been recently associated with a CVID phenotype with mood disorders. However, in this case, the causal relationship between the phenotype and the genetic variation requires an ad hoc study for a formal demonstration.

Taken together, our findings suggest that elevated polyclonal IgM levels with a normal CSR recombination, may be a warning sign for a B-cell disorder and should prompt clinicians to consider a LPD in the investigation of patients affected. Moreover, an in-depth characterization of such patients at molecular and functional level may lead to the identification of novel immunological pathways, paving the way to targeted therapy.

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**Authorship Contributions**

V.G., E.C., C.P. organized, collected and analyzed the data; V.G., C.P. wrote the manuscript; G.G., R.P., A.S., L.d.V., G.S., A.D., M.R.S., performed the experiments; V.Ma., V.Mo., contributed with samples from some patients; G.DM., C.S., performed whole exome sequencing; all authors reviewed and approved the manuscript.

**Disclosure of Conflict of Interest**

The authors declare no competing financial interests.



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**TABLE I | Clinical features of the HIGM-like patients**

| | P1 | P2 | P3 | P4 | P5 | P6 | P7† |
|---|---|---|---|---|---|---|---|
| **Demographic characteristics** | | | | | | | |
| Age | 16 | 49 | 9 | 45 | 8 | 15 | 15 |
| Sex | F | F | M | M | M | F | F |
| **Clinical features** | | | | | | | |
| **Infections** | | | | | | | |
| Bacterial | + | + | + | - | + | + | + |
| Opportunistic | - | - | - | - | - | - | - |
| Viral | +* | +** | - | - | - | - | - |
| **Lung disease** | | | | | | | |
| Bronchiectasis | + | - | - | - | - | - | - |
| Atelectasis | + | - | - | - | + | - | - |
| Interstitial lung disease | - | + | - | - | - | - | - |
| **Lymphadenopathy** | + | - | ++ | +/- | + | + | ++ |
| **Autoimmunity** | - | - | AIHA, TI | - | + | - | - |
| **Inflammatory disease** | - | + | - | - | - | - | - |
| Recurrent fever | - | + | - | - | - | - | - |
| Musculoskeletal involvement | + | + | - | - | - | - | - |
| **Liver and/or spleen enlargement** | + | - | + | + | + | - | - |
| **Cutaneous manifestations** | + | + | + | + | + | - | - |
| **Cancer** | NH-Lymphoma | MALT-Lymphoma | LLA | - | - | Hodgkin-Lymphoma | Diffuse large B-cell Lymphoma |
| **Other** | | | | Mood disorder | Behavioral disorder | Growth and pubertal delay; bone defects; Arnold Chiari syndrome | Growth and pubertal delay |

*AIHA indicates autoimmune haemolytic anemia; TI, immune thrombocytopenia; NH, non-Hodgkin; MALT, mucosa-associated lymphoid tissue; LLA, acute lymphoblastic leukemia;*
**P1 experienced a measles complicated by pneumonia*
***P2 experienced a chickenpox with severe ocular involvement*

**TABLE 2 | Immunological and molecular findings of the HIGM-like patients**

| | P1 | P2 | P3 | P4 | P5 | P6 | P7† |
|---|---|---|---|---|---|---|---|
| **Immunological features** | | | | | | | |
| IgG, mg/dl | 393 | 858 | 287 | 1500 | 294 | 565(IVIG) | 140 |
| IgA, mg/dl | 183 | 282 | 200 | 227 | 33 | 5 | 5 |
| IgM, mg/dl | 801 | 911 | 454 | 516 | 800 | 443 | 596 |
| **Lymphocyte absolute counts/ml** | 6.950 | 3.300 | 2.360 | 1.400 | 3.090 | 2100 | 2260 |
| **T-cell subsets** | | | | | | | |
| CD3+% | 72 | 79 | 70 | 86 | 74 | 90 | 95 |
| CD4+% | 31 | 57 | 30 | 48 | 40 | 26 | 14 |
| CD8+% | 31 | 21 | 28 | 36 | 30 | 62 | 70 |
| CD56+% | 4 | 14 | 9 | 6 | 6 | *NA* | *NA* |
| **B-cell subsets** | | | | | | | |
| CD19+% | 25 | 5 | 21 | 2 | 12 | 4 | 0 |
| CD19+CD27+IgM+ (IgM memory, % of CD19+) | 3 | 20 | 2.3 | 5 | 1.6 | *NA* | *NA* |
| CD19+CD27+IgM- (switched memory, % of CD19+) | 2.9 | 0 | 0.47 | 10 | 12.5 | *NA* | *NA* |
| **Genetic alteration** | | | | ITPKB c.146_147insA | | PIK3R1 c.1425+1G>T | |
| **Inheritance** | | | | *NA* | | *de novo* | |

*NA, not available*

**TABLE 3 | *In vivo* and *in vitro* Class Switch Recombination**

| | P1 | P2 | P3 | P4 | P5 | P6 |
|---|---|---|---|---|---|---|
| ***In vivo* CSR** | | | | | | |
| IgG anti-HbsAg | - | *ND* | + | - | - | *NA* |
| IgG anti-Measles | + | + | *NA* | *ND* | *ND* | *NA* |
| IgG anti-CMV | + | + | *NA* | + | + | *NA* |
| IgG anti-EBV | + | + | *NA* | + | + | *NA* |
| IgG anti-VZV | + | + | *NA* | + | + | *NA* |
| IgG anti-Rubella | - | *ND* | *NA* | + | *ND* | *NA* |
| IgG anti-Mumps | - | *ND* | *NA* | | + | *NA* |
| ***In vitro* CSR** | | | | | | |
| IgE pg/ml (not stimulated) | 173 | 2860 | *ND* | *ND* | *ND* | *ND* |
| IgE pg/ml (stimulated) | 14658 | 6120 | *ND* | *ND* | *ND* | *ND* |

*NA, not applicable; ND, not done*

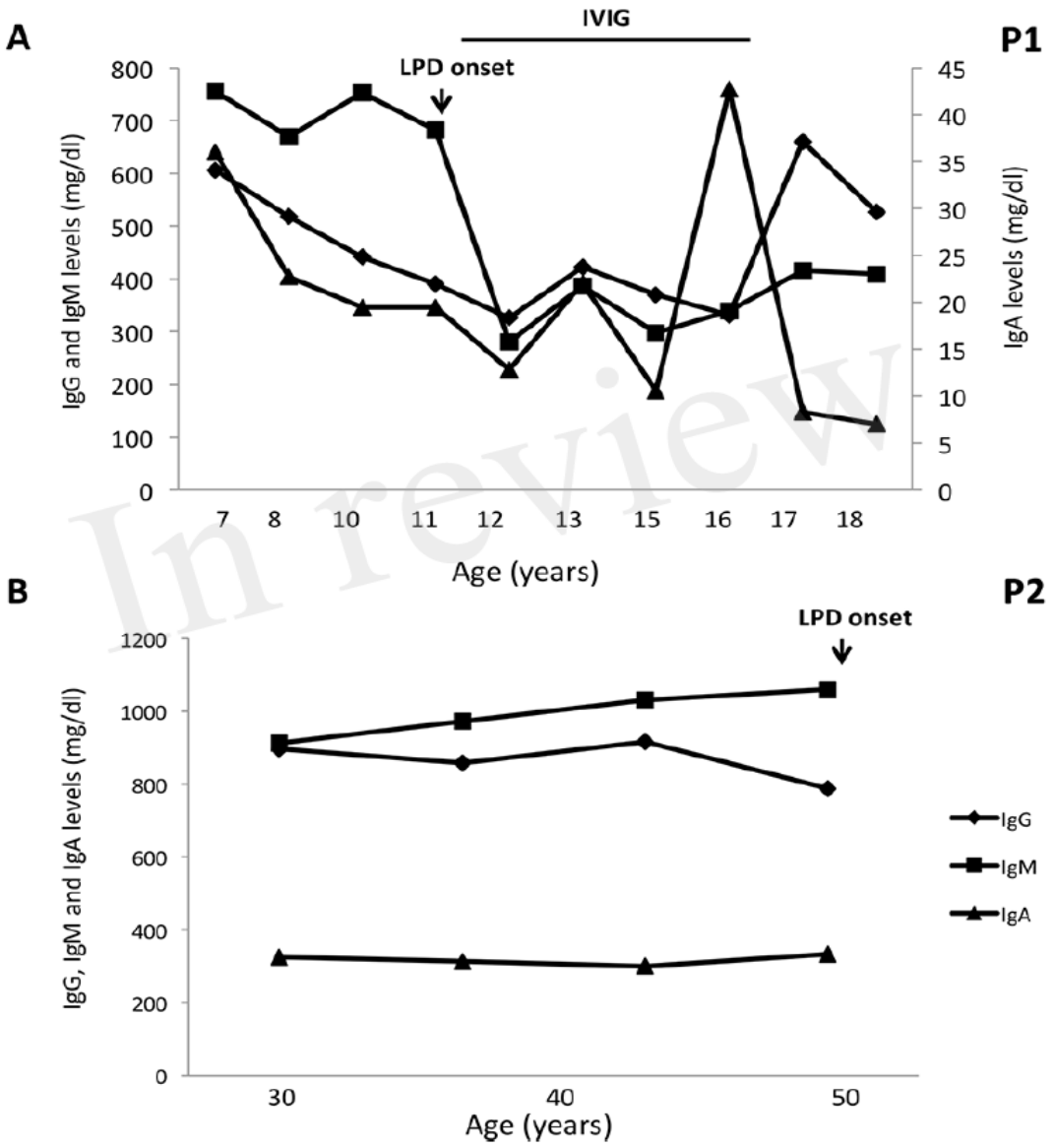


**Figure 1.** Long-term serum Ig levels evaluation. In P1 and P2 a long-term longitudinal evaluation of IgM levels documented elevated IgM levels 5 years before the onset of the LPD in P1 (A), and more than 10 years in P2 (B). No significant further increase in IgM levels was observed after the LPD diagnosis, whereas a significant reduction was documented in P1 in the subsequent 4 years post-chemotherapy, along with a reduction of IgG levels, requiring an Ig intravenous (IgIV) replacement therapy.

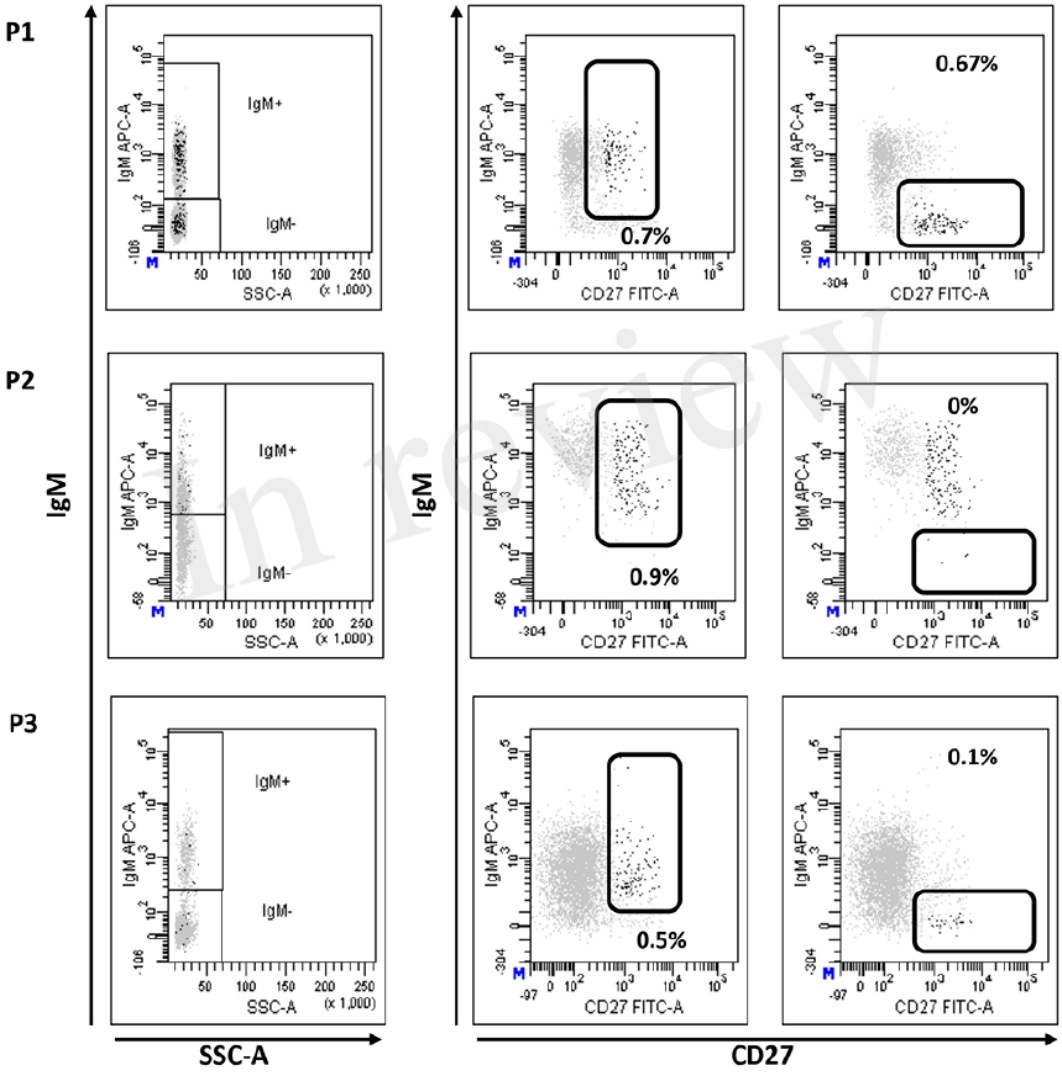


**Figure 2.** B-cell immunophenotyping. Representative flow cytometric plot showing IgM memory (CD19+ CD27+IgM+) and switched memory (CD19+ CD27+IgM-) expression in P1, P2, and P3. The B-cell subsets are expressed as percentage of absolute lymphocyte count.

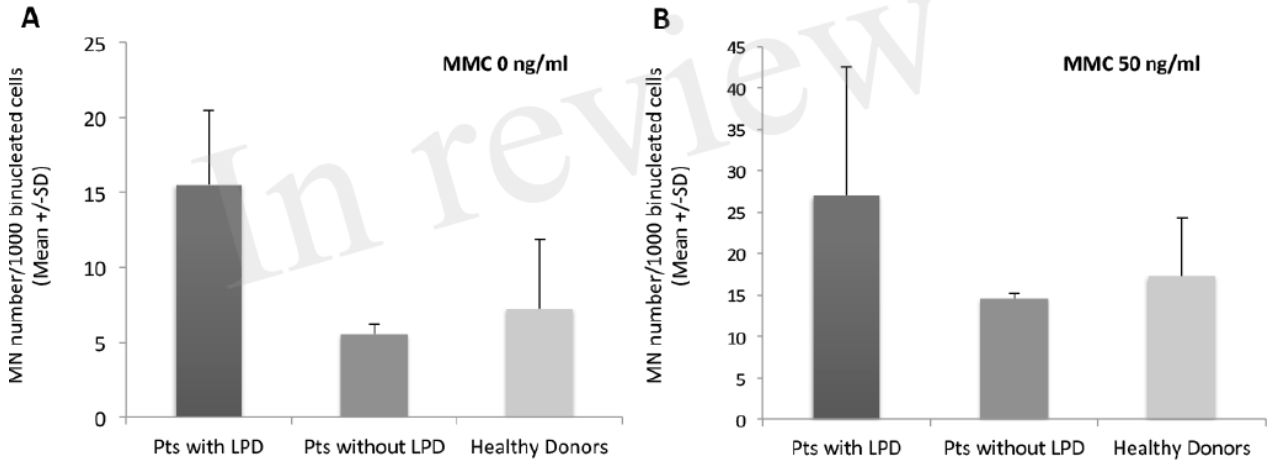




**Figure 3.** Spontaneous and chemically-induced DNA damage in patients and controls. Data are recorded in 4 patients, P1 and P2 (with LPD), P4 and P5 (without LPD) and four healthy donors. The frequency of MN was reported as MN number/1000 binucleated cells (mean value ± SD) in untreated (A) or treated (B) cultures with MMC concentration of 50 ng/ml.

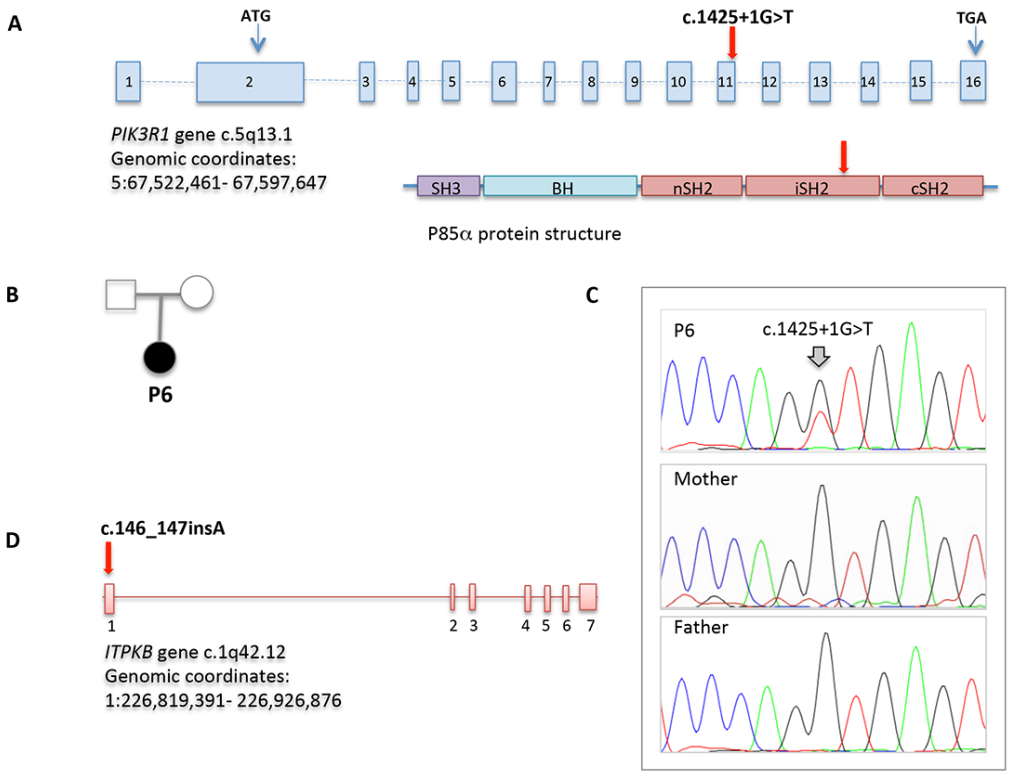


**Figure 4.** *PIK3R1* and *ITPKB* variations identified through whole exome sequencing. (A) *PIK3R1* gene structure which encodes p85α protein. The splice site mutation in patient 6 is indicated. (B) Pedigree of the family carrying the PIK3R1 mutation. (C) Sequencing chromatograms in patient and her parents. (D) *ITPKB* gene structure with the frameshift mutation identified in P4. Chromosome location and genomic coordinates are provided.

## CHAPTER III

# "New insights in Severe Combined Immunodeficiency and other T-cell disorders"

Severe combined immunodeficiencies (SCIDs) encompass a wide group of primary disorders due to defects in several genes involved in T- and often B-cell development or function, which result in severe and life-threatening infections. Typically, SCID patients have absent T cells and are further grouped on the basis of the absence or presence of B and NK cells (70, 71).

The phenotypic complexity and heterogeneity of SCIDs are responsible for the difficulty in their recognition and frequently lead to a significant delay in the diagnosis. Thanks to the availability of new high-throughput deep sequencing analysis technology, during the last two years several (EXTL3, BCL11B, DOCK2, LAT**)** new SCID related genes have been identified. This technology also led to identify new clinical phenotypes associated with well-known genetic defects, thus adding to the complexity of the recognition.

The difficulty in the recognition of these patients also relies on the fine line between patients with the more severe forms and those with the milder phenotypes, actually defined as combined immunodeficiencies (CIDs).

In addition, not infrequently, patients may exhibit extra-immunological manifestations, which may predominate in the presentation of the disease (72).

In a few cases, disorders caused by hypomorphic mutations in known SCID-causing genes may present in infancy with immuno-dysregulation features, which may prevail and obscure the increased susceptibility to infections. Eventually, even within the same family, subjects carrying the same mutation exhibit diverse clinical and immunological features, indicating the complexity of the pathogenesis also for monogenic disorders.

The real prevalence of SCID among children is unknown. The introduction of population-based newborn screening has revealed an incidence of ~1 per 58,000 live births in the United States higher than previously expected which was of 1: 100,000 (73). This discrepancy clearly indicates that in many cases the presenting phenotypes are masked and that the diagnosis is hard even to be suspected. Indeed, many infants

in settings without newborn screening succumb to infectious diseases without having been recognized as immunodeficient.

In addition to ordinary bacterial and viral pathogens such as *Streptococcus pneumoniae*, cytomegalovirus (CMV) and adenoviruses, infants with SCID are also susceptible to opportunistic organisms such as *Pneumocystis jiroveci*; patients can also develop severe, systemic and often fatal disease when given live vaccines for rotavirus, poliovirus or Bacillus Calmette–Guérin vaccine (70, 71, 74).

The first example of SCID due to mutations of gene not expressed in hematopoietic cells is the human Nude/SCID phenotype, which is characterized by the absence of a functional thymus, which results in a severe T-cell immunodeficiency, caused by alterations in the transcription factor FOXN1 gene.

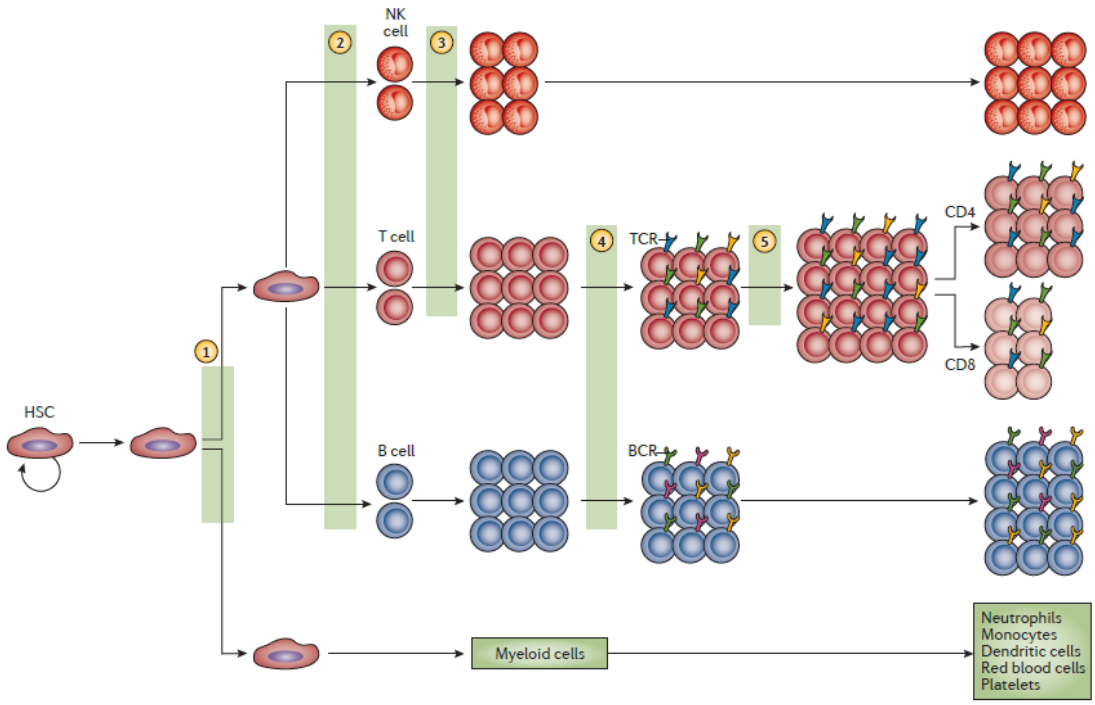


**Figure 7**. Representation of blocks in lymphocyte differentiation causing SCIDs (Fisher A, 2015).

Definitive treatment for patients affected with SCID is represented by allogenic HSCT, although gene therapy and enzyme replacement therapy are available for some specific genetic sub-type (75).

A review of the available literature, paying particular attention to the most recently identified forms and to their unusual or extra-immunological clinical features, has been published in *Annals of New York Academy of Sciencies*.

Furthermore, a new complex T-cell disorder in a child with a DiGeorge-like phenotype associated to a 3p12.3 deletion involving MIR4273 gene born to a mother with gestational diabetes has been accepted for publication in the *American Journal of Medical Genetics*.

In a further review published in *J Clinical Immunology* we summarize recent discoveries and novel therapeutic approaches for disorders of immune system with athymia such as FOXN1 deficiency.

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# Severe combined immunodeficiency—an update

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**Severe combined immunodeficiencies (SCIDs) are a group of inherited disorders responsible for severe dysfunctions of the immune system. These diseases are life-threatening when the diagnosis is made too late; they are the most severe forms of primary immunodeficiency. SCID patients often die during the first two years of life if appropriate treatments to reconstitute their immune system are not undertaken. Conventionally, SCIDs are classified according either to the main pathway affected by the molecular defect or on the basis of the specific immunologic phenotype that reflects the stage where the blockage occurs during the differentiation process. However, during the last few years many new causative gene alterations have been associated with unusual clinical and immunological phenotypes. Many of these novel forms of SCID also show extra-hematopoietic alterations, leading to complex phenotypes characterized by a functional impairment of several organs, which may lead to a considerable delay in the diagnosis. Here we review the biological and clinical features of SCIDs paying particular attention to the most recently identified forms and to their unusual or extra-immunological clinical features.**

**Keywords:** severe combined immunodeficiency; SCID; primary immunodeficiency; nude/SCID; DiGeorge syndrome; cytokine; thymus

## Introduction

Severe combined immunodeficiencies (SCIDs) are a group of inherited disorders responsible for severe dysfunctions of the immune system that lead to the absence or dysfunction of the T and B cells derived from the thymus gland and bone marrow, thus affecting both cellular and humoral adaptive immunity. Recently, Kwan *et al.*, on the basis of data obtained from 11 U.S. newborn screening programs in the general population, reported an incidence of SCID of 1 in 58,000 live-births, an incidence much higher than the previous estimate of one in 100,000 based on retrospective clinical diagnosis of SCID.[1] This group of diseases belongs to the most severe forms of primary immunodeficiency (PID), which are often fatal when the diagnosis is made too late.[2] Even though children with SCID appear healthy at birth, they are predisposed to severe bacterial, viral, and fungal infections as the maternal transferred antibodies decline. During the first year of life, failure to thrive, diarrhea, and oral candidiasis are common findings; *Pneumocystis jiroveci* may frequently cause a severe interstitial pneumopathy; and maternal engraftment of lymphocytes can cause graft-versus-host disease (GVHD).[3] SCID patients often die during the first two years of life if appropriate treatments to reconstitute their immune system are not undertaken.[4] For most patients, the only curative treatment is the allogeneic hematopoietic stem cell transplantation (HSCT).[5] Gene therapy offers a cure for two specific forms of SCID and, although other SCID forms may become amenable to this treatment in the future, it is likely that HSCT will continue to be used for the majority of SCID patients.[6]

Conventionally, SCIDs can be classified according either to the main pathways affected by the molecular defect or on the basis of the specific immunologic phenotype related to that genetic defect, as T cell–deficient but normal B cell ($T^-B^+$) SCID and both T cell– and B cell–deficient ($T^-B^-$) SCID, with a further subdivision depending on the presence or

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absence of NK cells ($NK^+$ or $NK^-$, respectively).[2] This classification, traditionally considered as representative of the stage where the blockage occurs during the differentiation process, was, until a few years ago, very useful in directing molecular studies toward a certain genetic alteration. However, during the last years many new causative gene alterations have been identified with peculiar clinical and immunological phenotypes. In a few cases, the genetic alteration allows for a normal T cell differentiation program but compromises T cell functionality by affecting the initial or final phase of intracellular signaling. These functional T cell disorders are characterized by immune dysregulation and cancer predisposition, as well as infections. In addition, hypomorphic mutations in some SCIDs genes make possible the development of nonfunctional oligoclonal T cells that are responsible for a complex of clinical conditions that may include hyperinflammation or autoimmunity. Many of the novel forms of SCID also show extra-hematopoietic alterations, leading to complex phenotypes characterized by functional impairment of organs different from primary lymphoid organs, which can make the diagnostic process very complex by standard methods. Taking this into account, the traditional international classification of SCIDs based on immunophenotype may no longer be optimal for clinical and research purposes[7,8]—diagnostic criteria have to be continuously updated to take into account these unusual phenotypic presentations. In his work of 2014, Shearer emphasizes that currently there is no consensus among clinical immunologists on how best to diagnose and treat these rare disorders. It is not surprising that an important clinical dilemma concerns the distinction of SCIDs from other diseases such as combined immunodeficiencies (CIDs). Recently, it was proposed that patients who exhibit an absence or a severe reduction of T cells ($CD3^+ < 300/\mu L$), absence or severe reduction (<10% of the lower limit) of a proliferative response to phytohemagglutinin, or a maternal lymphocyte engraftment should be defined as having typical SCID.[5] Moreover, the European Society for Immunodeficiency suggested as criteria for the diagnosis of CID the presence of one of the following parameters: one severe infection, an immunodysregulation disorder, cancer, familial CID associated with moderate age-related reduction of $CD3^+$, $CD4^+$, $CD8^+$ T cells or of naive T cells. However, a cutoff to distinguish SCID from CID has not yet been well defined.

A main aim of this review is to report on the biological and clinical features of SCID, paying attention to the most recently identified forms and to the unusual or extra-immunological clinical features (Table 2). An attempt to relate together pathogenetic mechanisms to specific clinical features is proposed (Table 1).

## SCID due to defective survival of hematopoietic lineage precursors

Reticular dysgenesis (RD) is an autosomal recessive form of SCID characterized by both early myeloid lineage differentiation arrest and impaired lymphoid development.[9] It is considered the most severe form of SCID, accounting for less than 2%. A peculiarity of this disorder is the presence of sensorineural deafness. RD is caused by biallelic mutations in the adenylate kinase 2 gene (*AK2*), which cause the absence or the strong reduction of the expression of AK2 protein.[9,10] The syndrome is characterized by the absence of granulocytes and lymphocytes in peripheral blood. Compared to all the other forms of SCID, RD-associated neutropenia, which is unresponsive to granulocyte-colony stimulating factor (G-CSF), predisposes the patients to severe infections.[11] The only available treatment for RD is allogeneic HSCT, which indicates that the inherited defect is cellular and not linked to the micro-environment, as previously thought. Neutrophil differentiation abnormalities of RD patients are corrected by the restoration of AK2 expression in the bone marrow, thus confirming the specific role of AK2 in the development of the myeloid lineage.[12] Moreover, AK2 is specifically expressed in the stria vascularis region of the inner ear, which explains the sensorineural deafness observed in these individuals.[10] AK2 is localized in the mitochondrial intermembrane space where it regulates adenine nucleotide interconversion within the intermembrane space;[13] a very similar function is mediated by the cytoplasmatic enzyme AK1. The function of AK1/2 is classically described to be the maintenance of a constant concentration of adenine nucleotides and the monitoring of mitochondrial energy state through a fine mechanism of nucleotide sensing and signaling. The molecule also plays a central role in the control of apoptosis through the Fas-associated protein with death domain (FADD)

**Table 1.** New clinical phenotypes associated with old forms of nonsyndromic SCID/CID and new genetic defects

| Gene defect | Old phenotype | New phenotype | Pathogenetic mechanism | Reference |
|---|---|---|---|---|
| *AK2* | Absence of granulocytes, severe lymphopenia sensorineural deafness | OS | Peripheral expansion of oligoclonal T lymphocytes | 15 |
| *IL2RG* (γc) | $T^-B^+NK^-$ SCID, leaky $T^+B^+NK^-$ | Hodgkin like features, invagination and HLH | Not clear; maternal GVHD | 55,56 |
| *JAK3* | SCID, immune-dysregulation and autoimmunity | Selective $CD4^+$ T lymphopenia | Hypomorphic mutation associated with somatic chimerism | 51 |
| *RAG* | Severe hypogamma-globulinemia, marked reduction of T and B cells, OS, incomplete OS | Granulomatous lesions, EBV-related lymphoma, Idiopatic $CD4^+$ T lymphopenia with extensive chickenpox | Hypomorphic mutations | 70 |
| *CORO1A* | $T^-B^-NK^+$ SCID, severe postvaccination chickenpox, language delay, behavioral and cognitive impairment | EBV B cell lymphoproliferation | Not clear; null and hypomorphic mutations of *Coro1A* in mice are associated with defects in T cell survival and migration | 79 |
| *FOXN1* | Human nude/SCID | Eczematous rash, erythroderma, severe diarrhea and alopecia | Residual T cell development sustained by rudimentary thymus or extrathymic lymphoid sites | 80 |
| *IL21R* | NA | Cryptosporidiosis, chronic cholangitis and liver disease, abnormal IL-21 induced proliferation, defect of immunoglobulin class-switching, and NK cell cytotoxicity | Abrogation of IL-21 ligand binding, defective cytokine secretion | 99 |
| *ZAP70* | Selective $CD8^+$ lymphopenia and normal/elevated numbers of not functional $CD4^+$ T cells | Late onset disease, cutaneous, erythematous lesions, immune dysregulation erythroderma | Possible role of hypomorphic mutations on T lymphocytes effector and suppressive function | 113 |
| *MALT1* | NA | CID | Abnormal IL-12 production, failure of IκBα degradation | 114 |
| *BCL10* | NA | Profound T and B memory cell deficiency, severe hypogammaglobulinemia | Impairment of NF-κB pathways | 115 |
| *CARD11* | NA | CID | Abnormal IL-12 production, $T_{reg}$ cells deficiency | 101 |
| *TTC7A* | NA | CID-MIA | Defective thymopoiesis | 116 |
| *LCK, UNC119* | NA | $CD4^+$ lymphopenia, restricted T cell repertoire, immune dysregulation | Impaired TCR signaling | 122 |
| *IKBK2* | NA | Mycobacterium avium and tuberculosis infections, neurological impairment, hypogammaglobulinemia, normal T cells count with absence of $T_{reg}$ and γ/δ T cells | Impairment of IKK2–NF-κB signaling | 124 |

Note: OS, Omenn syndrome; HLA, hemophagocytic lymphohistiocytosis, GVHD, graft versus host disease; MIA, multiple intestinal atresia; NA, not applicable.

**Table 2.** Pathogenetic mechanisms of SCID

| Pathogenetic mechanism | Defect | Phenotype | Inheritance |
|---|---|---|---|
| Defective survival of haematopoietic precursors | AK2 | $T^-B^-NK^-$ | AR |
| Toxic metabolite accumulation | ADA | $T^-B^-NK^-$ | AR |
| | PNP | $T^-B^+NK^-$ | AR |
| Cytokine signaling anomalies | IL-2RG | $T^-B^+NK^-$ | XL |
| | JAK3 | $T^-B^+NK^-$ | AR |
| | IL-7RA | $T^-B^+NK^+$ | AR |
| V(D)J recombination and TCR abnormalities | RAG1/RAG2, Artemis, DNA-PKcs, Cernunnos, LIG4 | $T^-B^-NK^+$ | AR |
| TCR abnormalities | CD45 | $T^-B^+NK^+$ | AR |
| | CD3ε, δ, ζ | $T^-B^+NK^+$ | AR |
| | CORO1A | $T^-B^-NK^+$ | AR |
| Thymic abnormalities | FOXN1 | $T^{-/low}B^+NK^+$ | AR |
| | DiGeorge sindrome | $T^-B^+NK^+$ | De novo or AD |

and caspase 10 pathways.[14] Omenn syndrome (OS), resulting from residual development and peripheral expansion of oligoclonal T lymphocytes, has recently been described in a patient with RD due to missense mutation in *AK2*.[15] OS is a clinical condition characterized by generalized skin rash, hepatomegaly, splenomegaly, lymphadenopathy (similar to that which occurs in SCID patients with detectable $CD3^+$ T cells), absent or low T cell proliferation to common antigens, and no maternal engraftment. Increased IgE serum levels and eosinophil count are also common features. In rare patients with RD, no mutations in *AK2* have been found, suggesting a potential role for other molecules involved in this pathway. For instance, a similar phenotype has been described in a murine models either deficient for growth factor independence-1 (Gfi-1) or transgenic for expression of Gfi-1b nucleoproteins, suggesting a role for these two factors in the pathogenesis of RD.[16]

## SCID due to accumulation of toxic metabolites

Adenosine deaminase (ADA) deficiency and purine nucleoside phosphorylase (PNP) deficiency are inherited disorders of the purine metabolism characterized by abnormal accumulation of toxic nucleoside products.[17] ADA deficiency is responsible for a T cell–, B cell–, and NK cell–deficient ($T^-B^-NK^-$) form of SCID associated with thymic hypoplasia and absence of lymphocyte proliferative response. Before the introduction of newborn screening, the incidence of this autosomal recessive disorder was estimated to be between 1:375,000 and 1:660,000 live births.[18] However, a recent trial on a population-based neonatal screening revealed that the incidence of ADA-SCID is much higher, and closer to 1:50,000.[19] The ADA gene of 12 exons is located in a 32 kb region on chromosome 20q13.11. Several genetic alterations, with more than seventy mutations, have been identified in ADA-SCID patients.[20] The product of *ADA* is an ubiquitous enzyme that catalyzes the irreversible deamination of adenosine (Ado) and deoxyadenosine (dAdo) to inosine and deoxyinosine, respectively. Despite ADA protein being present in virtually every cell of the human body, it is particularly expressed in the lymphoid system, especially in the thymus, where it plays a key role in its differentiation and maturation. The absence of ADA activity is responsible for a massive accumulation of Ado and dAdo, in particular in thymocytes, lymphocytes, and erythrocytes.[17,21] dAdo phosphorylation by nucleoside kinases leads to the production of deoxynucleotide triphosphates (dATP) whose accumulation, altering lymphocyte signaling pathways and serving as a danger signal, may cause the severe lymphopenia observed in ADA deficiency. Another alternative pathogenic mechanism proposed is the inhibition of *S*-adenosylmethionine–mediated transmethylation reactions required for

cell viability and normal differentiation.[22] By the first 6 months of age up to 80% of patients show multiple recurrent opportunistic infections that rapidly may become fatal and hypoplasia or apparent absence of lymphoid tissue. However, in the remaining patients, a late-onset phenotype, presenting at two or three years of life, or even later,[23] has been reported. These patients may also present with autoimmune diseases and usually exhibit a milder T cell immunodeficiency, which gradually progresses. Owing to its ubiquitous expression normally, ADA deficiency can affect several organs, leading to the development of skeletal alterations, such as anterior rib cupping, scapular spurring, and pelvic dysplasia, which can be reversible with appropriate therapy. In addition, pulmonary alveolar proteinosis, probably caused by a surfactant metabolism defect, and hepatic, gastrointestinal, and neurological disorders, mainly due to Purkinje cell damage, may be found. Bone marrow hypocellularity and myeloid dysplasia also have been observed in some ADA-deficient patients; in others, renal impairment.[24,25] A genotype–phenotype correlation has been documented and, in particular, severity of disease seems to correlate with residual ADA activity and the types of substrates that accumulate.[26] The therapeutic approach currently available for this particular form of SCID includes three options: enzyme replacement therapy with polyethylene glycol-modified bovine adenosine deaminase, HSCT, or gene therapy.[27–29] The use of dried blood spot samples tested by tandem mass spectrometry has been recently proposed as part of a neonatal program of screening in several countries.

Purine nucleoside phosphorylase gene (*PNP*) mutations result in an extremely rare autosomal recessive disorder accounting for 4% of all form of SCIDs.[30] Autoimmunity, recurrent infections, failure to thrive, and neurologic dysfunction are some of the main features of PNP deficiency. *PNP* maps to chromosome 14q13 and encodes a protein that catalyzes the phosphorolysis of guanosine, deoxyguanosine, inosine, and deoxyinosine, to their respective purine bases.[17,31,32] Mutations in the PNP pathways result in elevated deoxyguanosine triphosphate storage and in T cell toxicity due to the inhibition of the mechanisms of DNA synthesis and repair, resulting in an increased sensitivity to DNA damage and apoptosis, especially in T lymphocytes during selection within the thymus.[33] T cell defects typically become evident by the first year of life, with a milder phenotype than what is normally seen in ADA deficiency. PNP deficiency can be suspected when lymphopenia is associated with reduced PNP enzymatic activity in red blood cells in a patient with recurrent respiratory infections and other typical manifestations.[34] Low serum uric acid (hypouricemia) is usually found, although PNP deficiency should not be ruled out if patients do not exhibit it. The immunodeficiency in these patients is progressive, since the severe T cell deficiency usually appears after the second year of life and is characterized by a normal B cell compartment. Among the neurological disorders associated with PNP deficiency, ataxia, developmental delay, and spasticity have been described. Autoimmune diseases observed include hemolytic anemia and sclerosing cholangitis,[35] and in some patients megaloblastic or dysplastic bone marrow has been described.[33]

## SCID due to cytokine signaling anomalies

Cytokines are soluble regulators of immune system homeostasis. Alterations of their signaling are implicated in the pathogenesis of the major SCIDs. In particular, SCIDs caused by defects of the common gamma chain ($\gamma$c), Janus kinase 3 (JAK3), or the IL-7 receptor $\alpha$ chain (IL-7R$\alpha$) are prototypic cytokine-associated disorders, accounting for 67–74% of all cases of SCIDs.[36,37]

Mutations of $\gamma$c gene cause X-linked SCID (X-SCID), one of the most common forms of SCID, accounting for 50% of all cases. The $\gamma$c gene (*IL2RG*) maps to chromosome Xq13.1 and encodes a transmembrane protein that is a component of several cytokine receptors, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, all critical for lymphocyte development and function.[38] The $\gamma$c interacts with the intracellular tyrosine kinase JAK3, which acts as a transducing element[39] indispensable for cell growth and control of hematopoietic cell development. Evidence indicates that $\gamma$c is widely expressed in non-hematopoietic cells as well, even though its function in these cells has not yet been clearly elucidated. It has been reported that $\gamma$c is implicated in the growth hormone receptor signaling, suggesting the existence of a subtle interaction between endocrine and immune systems.[40–44]

JAK3, mainly expressed in lymphoid and myeloid cells, is essential for the differentiation of

hematopoietic precursors;[45–47] its deficiency is responsible for an autosomal recessive SCID. Molecular alteration of JAK3 may affect any of its functional domains and results in a $T^-B^+NK^-$ form of SCID, with a clinical phenotype similar to that observed in γc deficiency.[48] The immunological phenotype is due to the key role of γc/JAK3 signaling in both early T and NK cell, but not B cell, differentiation programs. However, B cell–intrinsic abnormalities, such as impaired class switch recombination and defective antibody production, have been documented. The identification of IL-7R–deficient SCID patients with a selective T cell defect[37] implies that the T cell defect observed in SCID due to mutations of γc/JAK3 results from defective IL-7 signaling. The ability of IL-15 to drive NK cell development[49] explains the lack of NK cells in γc/JAK3-deficient patients as a consequence of defective IL-15 signaling.[50] The molecular basis of the B cell functional abnormalities in patients with γc/JAK3 deficiency is probably linked to a defect in IL-21 secretion, a cytokine involved in proliferation, Ig isotype switching, plasma cell generation, and antibody secretion through activation of the JAK/STAT pathway.

Recently, hypomorphic mutations in JAK3 associated with somatic chimerism have been reported in a patient with predominant $CD4^+$ lymphopenia.[51] This observation suggests that hypomorphic mutations and/or somatic chimerism in other genes, which usually cause a SCID phenotype, eventually could be implicated in selective $CD4^+$ lymphopenia. Individuals with mutations that result in the production of a small amount of gene product or a protein with residual activity are less frequently seen. These individuals may have an atypical "leaky" disease characterized by $T^+B^+NK^-$ phenotype that is associated with immune dysregulation and autoimmunity, rashes, splenomegaly, gastrointestinal malabsorption, and/or short stature;[52,53] a few patients have presented with an OS phenotype,[54] which is characterized by elevated IgE, erythroderma, and an expansion of cells with a lymphocyte profile.

A peculiar extranodal lymphoproliferative disorder characterized by a polymorphous $CD20^+$ B lymphocyte infiltrate, resembling Hodgkin Reed-Sternberg cells, has also been observed in two patients affected with X-SCID.[55] Recently, a novel mutation in exon 5 of the γc gene has been reported that causes a classical severe immunological phenotype associated with invagination and hemophagocytic lymphohistiocytosis (HLH).[56] The HLH phenotype, previously described in two other cases with γc gene mutations,[57] is probably explained by maternal GVHD, and highlights the need for a fine-grained evaluation of the immunological phenotype and associated genotypes in patients with HLH.[58] As for the mechanism by which maternal engrafted T cells may be responsible for HLH in such cases, it is reasonable to hypothesize that unchecked T cell dysregulation of $CD8^+$ cells, activated by alloantigens, may result in cytokine hypersecretion and massive macrophage activation, eventually leading to hemophagocytosis.

The mutations of IL-7Rα gene (*IL7R*) cause a $T^-B^+NK^+$ SCID with an autosomal recessive transmission that is responsible for 10% of all SCIDs. The human *IL7R* maps to chromosome 5p13.2 and encodes for a protein[59] that is a component of two cytokine receptors, namely IL-7R and thymic stromal lymphopoietin receptor (TSLPR). Following the binding of IL-7 to IL-7R, JAK1 (coupled to IL-7Rα) and JAK3 are activated, which induces the phosphorylation of IL-7Rα, the recruitment of STAT5, and phosphatidylinositol 3-kinase (PI3K) at the receptor signaling apparatus. STAT5 molecules dimerize and translocate to the nucleus, leading to the transcription of IL-7–dependent genes. PI3K induces Akt activation, which prevents cell death through inhibition of Bad and regulates the kinase activity of Tor, eventually leading to the induction of several nuclear targets, including nuclear factor of activated T cells (NF-AT), NF-κB, and cyclin D1. Finally, activation of the Ras/MAPK/ERK pathway results in the induction of other nuclear targets, such as c-Myc, STAT1/3, and the Ets transcription factors. IL-7R is almost exclusively expressed by cells of the lymphoid lineage and is involved in thymocyte survival and maturation, particularly during $CD8^+$ positive selection.[60]

TSLPR, expressed mainly on monocytes, dendritic cells (DCs), and some types of T lymphocytes, is able to activate JAK2/STAT5 pathway, although this does not lead to cell proliferation. Human TSLP acts primarily on DCs, promoting DC-mediated expansion of $CD4^+$ T lymphocytes that acquire a memory T cell phenotype. The clinical phenotype of this form of SCID is quite heterogeneous and includes peculiar features such as OS,[61] cytopenia,[62]

severe and unresponsive cytomegalovirus (CMV) infection, or diarrhea of probable viral origin.[61]

## SCID due to V(D)J recombination and TCR abnormalities

V(D)J recombination is a complex process that occurs in early B and T cell development. It is responsible of the introduction of site-specific DNA double strand breaks (DSBs) by the recombination activating genes (RAG) 1 and 2.[63,64] The cleavage of the hairpin and the joining of these segments requires the DNA nonhomologous end-joining (NHEJ) DNA repair factors, which generate the diversity through recombination of the V, D, and J segments and junction.

NHEJ also plays a role in preserving the genomic stability of cells exposed to X-ray DNA damage. Consistent with these functions, it is not surprising that mice lacking NHEJ components exhibit a SCID phenotype and radiosensitivity (RS), a phenotype referred to as RS-SCID. In humans, several mutations in NHEJ genes have been identified, including mutations in genes for DNA ligase IV (*LIG4*), XLF/Cernunnos (*NHEJ1*), DNA-PKcs (*PRKDC*), and Artemis (*DCLREIC*), that are associated with SCID.[64–66] Of note, the increased radiosensitivity peculiar to these forms of SCID can be used as a diagnostic tool.[67,68]

Owing to the essential role of RAG1/RAG2 genes in V(D)J recombination, mutations of *RAG1* and/or *RAG2*, associated with partial protein expression and limited production of T and B cells, have been associated with a $T^-B^-NK^+$ SCID, OS, and autoimmunity.[69] Hypomorphic RAG gene mutations have also been described in patients with granuloma formation[70] and EBV-related lymphoma.[71] Since different clinical phenotypes have been associated with similar RAG mutations resulting in the same biological effect, a complex pathogenetic mechanism, based not only on the residual recombinase activity but also on the type and the moment of antigenic pressure has been postulated.

Artemis deficiency causes T cell maturation and B cell differentiation arrest at the pre-B cell checkpoint, resulting in a $T^-B^-NK^+$ SCID.[68] DNA-PKcs is involved in Artemis regulation and activation by both phosphorylation and complex formation, thus regulating enzymatic activities critical for V(D)J recombination.[64,72] Deficiency of DNA-PKcs causes a phenotype similar to Artemis deficiency.

The deficiency of XLF/Cernunnos causes a $T^-B^-NK^+$ SCID phenotype associated with microcephaly.[73] In particular, the phenotype is characterized by a progressive decrease of B cells and the presence of only memory T cells. Crystallography studies showed that XLF/Cernunnos is a component of the LIG4/XRCC4 complex, which exerts a role in aligning the two DNA ends in the DNA repair complex machinery. Deficiency of LIG4 is responsible for facial dysmorphisms, microcephaly, and variable forms of PID, ranging from SCID/OS to hypogammaglobulinemia or moderate defects in T and B cell functions.[74]

Gene mutations that abrogate early TCR signaling are associated with profound abnormalities of T lymphocyte development and function. CD45 (leukocyte common antigen) is a transmembrane tyrosine phosphatase involved in both TCR signaling and T cell development within the thymus and B cell development and maturation. CD45 deficiency is responsible for a very rare form of $T^-B^+NK^+$ SCID in which lymph nodes lack germinal centers.[75] Despite a normal monocyte numbers, T lymphocyte numbers are considerably decreased, with normal expression of TCR $\gamma\delta$ chains but a reduction of TCR$\alpha\beta^+$ cells. B cells, even though nonfunctional, are increased in number.

CD3 is a multimeric complex involved in TCR signaling and required for T cell differentiation. Defects of the complex can involve all the chains, resulting in a $T^-B^+NK^+$ phenotype. Alterations of the subunits epsilon (CD3$\varepsilon$), delta (CD3$\delta$), and zeta (CD3$\zeta$), have been reported in patients with severe forms of SCID, while alterations of the CD3$\gamma$ have been associated with a more benign course. These disorders are rare and inherited as autosomal recessive SCIDs. Some mutations can allow residual T cell maturation, even though the cross-talk between thymocytes and thymic epithelial cells may be impaired, thus compromising central tolerance and regulatory T cell ($T_{reg}$) development. Autoimmune manifestations, including autoimmune hemolytic anemia, vitiligo, Hashimoto's thyroiditis, autoimmune enteropathy, Evans syndrome, autoimmune hepatitis, and nephrotic syndrome are frequently observed in such patients.[76]

Coronin-1A is important for regulation of actin polymerization of cytoskeleton and essential for T cell migration from the thymus to the secondary lymphoid organs.[77] The human coronin-1A gene

(*CORO1A*) maps to chromosome 16p11.2 and encodes a highly conserved 57-kDa actin-binding protein expressed in both hematopoietic and immune cells. Coronin 1A–deficient neutrophils of mice have a normal adherence, membrane dynamics, migration, phagocytosis, and oxidative burst; dendritic cells are similarly not impaired. However, coronin 1A–deficient mice exhibit T cell lymphocytopenia and a normal number of B and NK cells, thus confirming its prominent role in T cell homeostasis and TCR signaling. In humans, deficiency of coronin 1A is associated with the absence of peripheral T cells.[78] However, different from other SCIDs due to other genetic alterations, a normal size thymus has been observed in the context of coronin 1A deficiency.[79] Hypomorphic *CORO1A* mutations have been associated with aggressive Epstein Barr virus–associated B cell lymphoproliferation, occurring at an early age.[79]

## SCID due to thymic abnormalities: from DiGeorge syndrome to nude/SCID

The prototype of athymic disorders caused by abnormalities of the stromal component of the thymus—the primary lymphoid organ for T cell differentiation—is the nude/SCID syndrome, described in humans in 1996.[80] This form of SCID is the only one not primarily related to an intrinsic abnormality of the hematopoietic cell, but rather to a defect in hematopoietic cell–supporting thymic epithelial cells.[81–83] This human SCID is the equivalent of the murine nude/SCID phenotype described in 1966, although in humans the phenotype is more severe. It is one of the rarest forms of SCID, and only three mutations have been associated thus far with nude/SCID.[84] The gene responsible for the disease in humans is *FOXN1*, located on chromosome 17,[85] which encodes a member of the forkhead/winged helix class proteins; this same gene is mutated in the same type of SCID in mice and rats. Forkhead/winged helix proteins is a large family of transcriptional factors implicated in several biological processes governing development, metabolism, cancer, and aging. FOXN1 is mainly expressed in the epithelial cells of the skin and thymus, where it plays a role in maintaining the balance between growth and differentiation. Thymic epithelial cell precursors require FOXN1 for full differentiation into cortical and medullary thymic epithelial cells capable of supporting T cell development. In epithelial cells, FOXN1 contributes to keratinocyte proliferation and differentiation in hair follicles, and to the development of the choroid plexus epithelium; this could explain the major features that characterize patients with nude/SCID, namely the absence of the thymus, with a severe T cell defect (though normal B and NK cells) and abnormal skin development, including congenital alopecia and nail dystrophy. The syndrome belongs to the $T^-B^+NK^+$ subgroup of SCIDs.[81] Usually, there is a significant reduction of $CD3^+CD4^+$ T helper lymphocytes, while the number of $CD3^+CD8^+$ T cells is less reduced. Functionally, there is a severe impairment of the proliferative response to mitogens, as found in the other forms of SCIDs.

The mutations described in nude/SCID cause a complete absence of functional FOXN1 protein. The first known mutation identified in humans, R255X, truncates the protein before the start of the forkhead domain, while a second mutation, R320W, leads to a substitution in the protein's DNA binding domain. A third mutation, c.562delA, results in a frameshift and premature truncation of the protein (p.S188fs) after the first 24 amino acids of the forkhead domain. The disease is inherited as an autosomal recessive trait. Heterozygous patients show minor ectodermal anomalies, such as nail dystrophy and, in particular, leukonychia or koilonychia (spoon nail).[86,87] Recent studies support a role for FOXN1 as cofactor in the development and differentiation of the central nervous system.[88]

Bone marrow transplantation (BMT) to treat this nude/SCID, despite the favorable clinical course, often results in a progressive decline of the $CD4^+$ T cell compartment[89] owing to the fact that a normal thymus is necessary for the generation of the $CD4^+$ naive subset. Conversely, the production of $CD8^+$ naive lymphocytes after BMT is less thymus dependent and even occurs in nude/SCID patients. In addition, a recent study showed the presence of T lymphocytes in a $FOXN1^{-/-}$ human fetus, suggesting partial T cell ontogeny in a thymus- and FOXN1-independent process.[90] Thymus transplantation has been shown to lead to immune reconstitution in two nude/SCID patients affected with disseminated Bacillus Calmette-Guérin infection and cytopenia.[91]

Before the identification of human nude/SCID, the DiGeorge syndrome (DGS) was long considered the model of a severe T cell differentiation defect. DGS is a complex disorder that typically

comprises T cell deficiency due to thymic hypo/aplasia, hypoparathyroidism, conotruncal cardiac defects, facial abnormalities, cognitive defects, speech delay, other birth defects, and gastrointestinal disorders.[92] Deletion of 22q11.2 is the most frequent chromosomal change associated with DGS,[93] with an incidence of one in 4000–5000 live births. The alteration is inherited in a familial autosomal dominant pattern in 8–28% of the cases.[94] Most patients have a deletion of 3 Mb that includes about 30 genes, while in 8% of the cases a smaller deletion of 1.5 Mb containing 24 genes is detected. No specific genotype–phenotype relationship has been documented. Both deletions include the gene T-box transcription factor 1 (*TBX1*), which seems to be necessary for normal development of the thymus and parathyroid, the large arteries of heart, and the muscles and bones of face and neck. Thymic hypoplasia, responsible for the thymic dysfunction, is observed in more than 80% of patients. The syndrome may be associated with variable T cell deficiencies, ranging from close to normal T cell numbers and functions, to complete DGS with a $T^-B^+NK^+$ SCID-like phenotype accounting for less than 1% of DGS.[95] Recently, a phenotype characterized by a $T^-B^-NK^+$ SCID has been described in two DGS patients with a concomitant Artemis deficiency.[96] Patients with complete DGS, like other infants with SCID, suffer from severe opportunistic infections and exhibit a high risk of acquired GVHD if transfused. Furthermore, a few patients affected with an atypical complete DGS have mature T cells derived from maternal engraftment or oligoclonal expansion of memory T cells responsible for a severe inflammation. These patients may develop an OS, characterized by erythrodermia, enteropathy, and lymphadenopathy. On the other hand, there are also subjects carrying the deletion who only have a mild phenotype. Some patients diagnosed as 22q11.2DS in early childhood remain clinically asymptomatic and exhibit only minimal immune alterations. Increased prevalence of atopic and autoimmune diseases has been reported in patients with partial deletion syndrome.[97] While normal B, NK, and T cell numbers are frequently observed in 22q11.2DS individuals, sometimes, a decrease of $CD4^+$ and $CD8^+$ T lymphocytes may be found[97] due to lower thymic output of the naive T cell subset, oligoclonal T lymphocyte expansion,[98] or altered T cell differentiation. These observations can be explained by the dysregulation of peripheral T cell homeostasis due to a defect in IL-7 signaling, crucial for T lymphocyte survival and expansion and for homeostasis of the naive $CD4^+$ T cell pool. Indeed, subjects with 22q11.2DS show a significant decrease of $CD3^+$ T lymphocytes expressing IL-7Ra; adults have accelerated conversion of naive to memory cells, shorter telomeres, and a defect in the variability of the TCR repertoire.[98]

A DGS phenotype has been described in patients carrying a 10p deletion, the clinical features being almost undistinguishable from 22q11.2DS. Even though low numbers of T cells, reduced immunoglobulin,[80] and thymus hypoplasia have been observed in 28% of such patients, none have been affected with a severe SCID-like phenotype.[95]

## SCID/CIDs associated with syndromic features

According to the International Union of Immunological Societies (IUIS), there are forms of PID associated with highly pleomorphic extra-immunological features responsible for complex syndromes with a genetic basis. Typical features of these syndromes comprise peculiar facial dysmorphism, growth delay, microcephaly, and ectodermal abnormalities. While an increased susceptibility to autoimmunity and (occasionally) cancer associated with the depletion of other blood cell lines is frequently reported, an increased susceptibility to infections is usually less frequent, and its clinical relevance is lower than in other PIDs. The pathogenetic mechanism resides in the involvement of several genes expressed in multiple cell lines, genes responsible for both ontogenesis and maturation of the immune system, as well as morphogenesis and organogenesis of other organs. Some of these conditions may be associated with a SCID/CID phenotype. Several syndromes are included in this group (Table 3), such DGS and CHARGE syndrome. Patients with CHARGE syndrome exhibit variable grades of immune defects, ranging from severe to mild T cell lymphopenia and abnormal T cell functionality, sometimes associated with hypogammaglobulinemia.[102] The incidence of SCID in patients with CHARGE is unknown, even though it may be, as in DGS, rare.[103] These patients, whose clinical phenotype is characterized by coloboma, heart defect, atresia choanae, retarded growth and development, genital hypoplasia, and

**Table 3.** Peculiar clinical and laboratory findings in the main genetic syndromes which in a few cases may be associated with a SCID/CID phenotype

| Disorder | Genetic defect | Clinical phenotype | Immunological features |
|---|---|---|---|
| CHARGE syndrome | *CHD7* | Coloboma, hearth defect, atresia choanae, retarded growth and development | $T^-B^+NK^+$ SCID, OS, T cell lymphopenia, hypogammaglobulinemia |
| Cartilage–hair hypoplasia (CHH) | *RMRP* | Short limb with metaphyseal dysostosis, sparse hair, neural dysplasia of intestine | T cell lymphopenia, hypogammaglobulinemia, antibody deficiency |
| Schimke immuno-osseous dysplasia | *SMARCAL1* | Short stature, IUGR, spondiloepiphyseal dysplasia | T cell lymphopenia, bone marrow failure |
| Hyper IgE syndrome | *PGM3* | Short stature, brachydactyly, facial dysmorphism, intellectual disability | Congenital leucopenia, neutropenia, B and T cell lymphopenia |
| Hoyeraal-Hreidarsson syndrome (HHS) | *DKC1* | Microcephaly, cerebellar hypoplasia, IUGR | Bone marrow failure, CID or $T^+B^-NK^-$ SCID |
| Folate and cobalamin metabolism defect | *PCFT, TCN2, MTHFD1* | Failure to thrive, weakness, mental retardation, megaloblastic anemia, neurological disease | Pancytopenia, SCID-like phenotype, hypogammaglobulinemia |
| Anhydrotic ectodermic dysplasia with immunodeficiency | *NEMO* | Hypohidrosis, hypodontia, conical teeth, facial dysmorphism | SCID/CID-like phenotype |

IUGR, intrauterine growth restriction.

ear anomalies/deafness, may suffer from a $T^-B^+$ $NK^+$ SCID and, in some cases, OS.[103] The disorder is caused by mutations in the chromodomain helicase DNA binding protein 7 gene (*CHD7*), a member of the chromo domain helicase DNA binding domain family of adenosine-5′-triphosphate dependent chromatin remodeling enzymes. CHD7 is expressed throughout the neural crest containing mesenchyme of the pharyngeal arches, suggesting a pathogenetic overlap between CHARGE and DGS.

In other syndromes, several peculiar skeletric abnormalities are the main feature, which lead the patient to the medical attention, as observed in patients with cartilage–hair hypoplasia (CHH), characterized by severe disproportionate short stature due to short limb with metaphyseal dysostosis, sparse hair and neural dysplasia of the intestine,[104] or in Schimke immuno-osseous dysplasia, which sometimes may show a CID phenotype.

In humans, defects in gene involved in telomere maintenance (*TERT*, *TERC*, *DKC1*, *WRAP53*/*TCAB1*, *NOP10*, *NHP2*, and *TINF2*) are responsible for the dyskeratosis congenita (DC), a rare congenital disorder characterized by progressive bone marrow failure, premature aging, mucocutaneous abnormalities, and cancer predisposition.[106] The most sever infantile variant of X-linked DC is the Hoyeraal-Hreidarsson syndrome (HHS), whose main clinical features are microcephaly, cerebellar hypoplasia, and intrauterine growth retardation. The early-onset bone marrow failure usually leads to either a combined immunodeficiency or a $T^+B^-NK^-$ SCID, which may require HSCT.[107]

Recently, several inborn errors in folate and cobalamin metabolism have been described as having a profound impact on many systems, including hematopoiesis and neuronal function. Immunodeficiency of variable degrees has been associated with defects in these pathways. A CID phenotype

characterized by lymphopenia, responsiveness to folate replacement therapy, and severe bacterial and viral infections has been described in patients with functional methionine synthase deficiency caused by hereditary folate malabsorption due to deficiency in the proton coupled folate transporter (PCFT) and in transcobalamin II (TCN2); this CID usually presents in early infancy in untreated patients as failure to thrive, weakness, pancytopenia, and intellectual disability. Recently, exomic sequencing demonstrated that heterozygous mutations in the trifunctional protein MTHFD1 is responsible for a SCID-like phenotype characterized by $T^-B^-NK^-$ lymphopenia, marked hypogammaglobulinemia, megaloblastic anemia, and neurologic disease.[108] A partial immune reconstitution after vitamin B12 and folate replacement therapy has been documented.

In summary, it must be noted that several syndromes, together with the more typical severe manifestations, can share clinical and immunological signs of SCID/CID, as for example patients affected by NEMO deficiency.

## Recently identified combined immunodeficiencies

Combined immunodeficiency (CID) is a group of genetic heterogeneous disorders characterized by severe recurrent infections, moderate reduction of T and B lymphocytes, and impaired cellular and humoral functionality that may reflect late defects in T cell development and function.[109,110] In most cases, it is not always easy to distinguish between patients affected with more severe forms and those with CID. Furthermore, a greater difficulty in making a clear classification is due to the fact that many inborn defects, which underlie these immune disorders, have recently been associated with both SCID and CID, in particular hypomorphic mutations. Several genetic defects responsible for a wide number of clinical conditions are comprised in this group (Table 2).[111] Besides the well-known genetic defects responsible for MHC class I (*TAP1*, *TAP2*, *TAPBP*) or class II deficiency (*CIITA*, *RRFX5*, *RFXAP*, *RFXANK*) associated with a predominant $CD8^+$ or $CD4^+$ selective deficiency respectively, the very rare *CD8A* defects and many others (see the new International Union of Immunological Societies classification, Ref. 111) have been identified recently. Since the number of these conditions is large, we have chosen to discuss only the most common form associated with new phenotypes and novel ones reported over the past 3 to 4 years.

ZAP70-related immunodeficiency is inherited in an autosomal recessive manner. It is caused by abnormal TCR signaling, which leads to a selective absence of $CD8^+$ T cells and normal or elevated numbers of non-functional $CD4^+$ T cells. ZAP70 has a key role in both mature T cell signaling and differentiation of thymic precursors. Finally, in some patients peculiar phenotypes have been observed. In particular, some patients exhibit an attenuated phenotype with a late onset disease and preserved production of $CD4^+$ T follicular helper ($T_{FH}$), T helper type I ($T_H1$), $T_H17$, and $T_{reg}$ cells. Immune dysregulation and severe erythroderma resembling OS have also been described, characterized by skin infiltrative lesions with activated $CD4^+$ T cells in the peripheral blood.[113]

Thanks to next generation sequencing technologies, which have provided a powerful tool to identify the molecular cause of PIDs of unknown genetic origin, new defects have been detected, even though in most cases the genetic cause still remains unknown.

Whole-exome sequencing recently demonstrated the presence of deleterious mutations in the phosphoglucomutase 3 gene (*PGM3*) in three unrelated subjects with recurrent infections, congenital leukopenia, neutropenia, B and T cell lymphopenia, and progression to bone marrow failure due to a congenital disorder of glycosylation (CDG). Two of the three children also had skeletal anomalies characterized by short stature, brachydactyly, dysmorphic facial features, and intellectual disability.[105] Thanks to this technology, Kotlarz *et al.* identified in 2013 two distinct homozygous loss of functions mutations in the interleukin-21 receptor gene (*IL21R*) in two unrelated children affected with cryptosporidiosis, chronic cholangitis and liver disease, recurrent upper and lower airway infections, and failure to thrive.[99] IL-21R binds to common $\gamma c$ and signals via JAK/STAT pathways.[100,101] The authors observed that the mutation was responsible for the aberrant trafficking of the IL-21R to the plasma membrane and for the abrogation of IL-21 ligand binding. These molecular alterations lead to defective phosphorylation of STAT1, STAT3, and STAT5. The immunophenotype of these patients was normal, but abnormal proliferation induced by

IL-21 and defects in immunoglobulin class-switching in B cells and NK cell cytotoxicity were documented. A defect in T cell secretion of several cytokines, including $T_H17$-associated cytokines IL-17F and IL-22, was reported, thus putatively explaining the increased susceptibility to cryptosporidial infection in these patients.

In the last few years mutations in the CARD9–BCL10–MALT1 (CBM) complex involved in NF-κB signaling have been associated with PID. In particular, autosomal recessive mutations in MALT1 gene have been described in patients with CID and severe bacterial, fungal and viral infections.[114] The MALT1-deficient T cells are not able to degrade IκBα or produce IL-2 following T cell activation. BCL10 has a role in several immune pathways critical for the function of the innate and the adaptive immune systems, and for the response to bacterial and fungal infections. Mutations in *BCL10* and other genes encoding for proteins interacting with MALT1, such as *CARD11* and *CARD9*, have also been recently described. Patients with BCL10 deficiency show a profound defect of memory T and B cells and severe hypogammaglobulinemia, with a reduction of CD69 and CD25 percentages and ICOS levels.[115] Even though CARD9 deficiency has been shown to selectively compromise defenses toward a limited number of fungal infections, mutations in CARD11, which plays a crucial role in the differentiation of both neuronal and immunologic tissues as a scaffold protein, are associated with a more profound CID characterized by abnormal T cell proliferation to anti-CD3/CD28 stimulation, expansion of late transitional B cells, mature B cells deficiency, and hypogammaglobulinemia.[101] Furthermore, CARD11-deficient T cells do not produce normal amounts of IL-2 or upregulate the IL-2 receptor α chain (CD25) after TCR stimulation, which contributes to $T_{reg}$ cell deficiency in these patients.

Mutations in tetratricopeptide repeat domain 7A (TTC7A), a member of the large family of proteins containing the tetratricopeptide repeat (TPR) domain, have recently been found in patients affected with CID and multiple intestinal atresia (MIA).[116] MIA is a clinical condition that can be isolated or may occur in association with variable grades of immunodeficiency ranging from SCID to a mild decrease of T cells and partially preserved thymic function. However, in all these genetic forms, profound $CD8^+$ T cell lymphopenia, reflecting the impaired cellular immunity and the defective thymopoiesis, has been observed. Severe hypogammaglobulinemia is also frequent. A higher frequency of bloodstream infections due to intestinal microbes has also been reported.

The clinical and immunological phenotypes of Ras homolog family member H gene (*RHOH*) deficiency is characterized by naive $CD4^+$ T cell deficiency, absence of recent thymic emigrants, increased number of effector memory T cells, restricted T cell repertoire, and reduced *in vitro* proliferation via CD3 stimulation.[117] Expressed mainly in hematopoietic cells, RhoH is a small GTPase that mediates interaction between Zap70 and LCK. RhoH deficiency determines both alterations in pre-TCR–mediated signaling and in positive selection, as observed in Zap70 deficiency. Expansion of memory T cells has also been observed in other CIDs, such as deficiency of DOCK8 or MST1. DOCK8 deficiency is an autosomal recessive form of CID associated with a hyper-IgE phenotype. Viral infections (especially of the skin) and malignancies are very common. Lymphopenia of $CD4^+$ and $CD8^+$ T cells, or predominantly $CD4^+$ lymphocytes, may be found. In addition, DOCK8 deficient patients exhibit defective differentiation of $T_H17$ cells and a reduction of B lymphocytes.[118]

The lymphocyte specific kinase LCK is involved in the initiation of signaling from the TCR [121] through the adaptor protein unc-119 lipid binding chaperone (UNC119). Recently, mutations in LCK or UNC119, which impairs LCK activation and signaling, have been identified. Main features of this phenotype include $CD4^+$ T cell lymphopenia, a restricted T cell repertoire, and impaired TCR signaling.[122] Patients with LCK deficiency frequently present with immune dysregulation and autoimmunity. Mutations in the magnesium transporter protein1 gene (*MAGT1*) result in a CID phenotype characterized by $CD4^+$ lymphopenia and abnormal T cell proliferation, which are responsible for chronic viral infections and EBV-related lymphoma, respectively.[123] Recently, a CID was observed in four unrelated patients with mutation of inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (*IKBKB*); the patients had severe bacterial, viral, fungal, mycobacterial infections associated with failure to thrive and neurological impairment. The immunological phenotype was

characterized by a/hypogammaglobulinemia and absence of $T_{reg}$ and $\gamma/\delta$ T cells. Even though T cell counts were normal, all the patients exclusively showed naive T and B lymphocytes.[124]

## Newborn screening for SCID

Recently, T cell receptor excision circles (TREC)–based newborn screening has been implemented in several countries. Compared with patients identified by the clinical features, patients identified through newborn screening programs, similar to children identified because of a positive familial history, can receive an early and accurate diagnosis by one month of life and then undergo HSCT or gene therapy by 3 months of age, before the occurrence of severe complications. This results in a significantly improved outcome.[125,126] The TREC assay, based on the detection of intracellular accumulation of products derived from process of T cell receptor gene splicing and rearrangement, is able to detect several defects, which result in either SCID or profound T cell lymphopenia that is also seen in patients affected with 22q11.2DS, CHH, CHARGE, and AT.[127] However, one limitation of the TREC assay is that it is not able to identify all forms of CID or atypical SCID. Some genetic disorders, such as deficiency of ZAP70, late onset ADA, Nijmegen breakage syndrome, MHC class II deficiency, and many others, are likely to be missed because TRECs are usually found at normal levels. The identification of kappa-deleting recombination excision circles (KREC), a sensitive marker of newly formed B cells, increases the possibility of identifying other forms of SCID/CID that are associated with low numbers of B lymphocytes, such as NBS and late onset ADA. Furthermore, it has been reported that tandem mass spectrometry can easily identify abnormal purine metabolites in newborns with typical or late onset ADA and PNP deficiency,[19] thus increasing the spectrum of disorders detectable through newborn screening.

## Conclusions

SCIDs are a heterogeneous group of syndromes related to alterations of distinct genes that cause abnormalities in the maturation and/or function of T, B, and/or NK cells. Recently, advances in next generation DNA sequencing have allowed new gene identification through whole exome sequencing or whole genome sequencing of several forms of SCID and CID of unknown cause. The phenotypic and the molecular heterogeneity of SCIDs, as revealed by the expanding phenotypes observed, is making traditional classification of this group of disorders very intricate. Frequently, different mutations in the same gene can lead to different clinical phenotypes, such as OS, leaky SCID, or CID, that may even be inherited with different mechanisms.

In this review we have focused in detail on different forms of SCID and CID, paying attention to the distinctive peculiar clinical and laboratory features, in order to provide information to clinicians for recognizing and carefully managing these novel forms of PIDs.

## Conflicts of interest

The authors declare no conflicts of interest.

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CLINICAL REPORT

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# DiGeorge-like syndrome in a child with a 3p12.3 deletion involving MIR4273 gene born to a mother with gestational diabetes mellitus

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Chromosome 22q11.2 deletion is the most common chromosomal alteration associated with DiGeorge syndrome (DGS), even though this is not the only underlying cause of DGS. In rare patients, mutations in a single gene, *TBX1*, have been described resulting in a DGS phenotype. Recently, it has been reported that at least part of the *TBX1* mutant phenotype is due to excessive bone morphogenetic proteins (BMP) signaling. Evidence suggests that miRNA may modulate the expression of critical T-box transcriptional regulators during midface development and Bmp-signaling. We report on a 7-year-old Caucasian male born to a mother affected with gestational diabetes (GDM) who had a 371Kb-interstitial deletion of 3p12.3 identified by array CGH, involving the *ZNF717*, *MIR1243*, and *4273* genes. The child presented with a DiGeorge anomaly (DGA) associated with unilateral renal agenesis and language delay. The immunological evaluation revealed a severe reduction and impairment of T lymphocytes. FISH analysis and *TBX1* sequencing were negative. Among the miRNA-4273 predicted target genes, we found *BMP3*, which is involved in several steps of embryogenesis including kidney and lung organogenesis and in insulin gene expression. Since, DGA is not commonly found in newborns of diabetic mothers, we hypothesize that the pathogenesis of DGA associated with GDM is multifactorial, involving both genetic and/or epigenetic cofactors.

**KEYWORDS**
3p12.3 deletion, array-CGH, DiGeorge syndrome, miRNA, rare genetic syndromes

## 1 | INTRODUCTION

DiGeorge syndrome (DGS), also known as 22q11.2 deletion syndrome (22q11.2 DS) was first described in the 1960s and classically comprises facial anomalies, hypoparathyroidism, cardiac malformations, developmental, and speech delay, and mild to moderate immune deficiency related to thymic a/hypoplasia (Cancrini et al., 2014). Chromosome 22q11.2 deletion is the most common chromosomal alteration associated with DGS, occurring in approximately 1:4,000 live births (Cirillo et al., 2014; Tezenas Du Montcel, Mendizabai, Ayme, Levy, & Philip, 1996). However, a small number of patients affected with other genetic syndromes share a few clinical features with the 22q11 spectrum, including Opitz G/BBB (McDonald-MCGinn et al., 1995; Robin, Opitz, & Muenke, 1996) and CHARGE syndrome (Devriendt, Fryns, Mortier, van Thienen, & Keymolen, 1998), and other chromosomal deletions as 16p11.2, 10p13, 17p13, 4q34.1q35.2 (Ballif et al., 2007; Cuturilo et al., 2011; Greenberg, Elder, Haffner, Northrup, & Ledbetter, 1988; Pignata et al., 1996).

Teratogenic influences such as maternal diabetes (Wilson et al., 1993) or prenatal exposure to retinoic acid or alcohol may also lead to a DiGeorge anomaly (DGA). However, only a minority of newborns of diabetic mothers have a DGA, thus implying the requirement for multiple factors for the expression of this anomaly. In rare patients with a DGS phenotype but without the 22q11.2 deletion, mutations in the single T-box (*TBX1*) gene, which plays an important role in regulating the expression of several transcription factors, have been

  

described (Yagi et al., 2003). However, in some patients with a presumed diagnosis of DGS, the underlying etiology cannot be identified (Rope, Cragun, Saal, & Hopkin, 2009). Chromosomal microarray analysis has been advocated as a first-line diagnostic approach for patients with multiple congenital anomalies, including patients with a phenotype suggestive of 22q11.2 DS and normal fluorescent in situ hybridization (FISH) (Busse et al., 2010).

We report on a child born to a mother with gestational diabetes mellitus (GDM) affected with a DGA, associated with a 3p12.3 deletion, involving the *ZNF717*, *MIR1243*, and *4273* genes, which have a role in the regulation of embryogenesis. Among the miRNA-4273 predicted target genes, there is the bone morphogenetic protein-3 (*BMP3*), which is involved in several steps of embryogenesis, including kidney and lung. We hypothesize that this alteration may act as a genetic cofactor in favoring the clinical expression of the DGA in newborns of diabetic mothers.

## 2 | CLINICAL REPORT

The proband is a 7-year-old Caucasian male referred to our Immunodeficiency Center at the age of 25 months because of recurrent upper respiratory infections (URIs), hypoparathyroidism, unilateral renal agenesis diagnosed during fetal life and language delay. He was the third child of non-consanguineous parents. The family history was unremarkable except for a maternal aunt, affected with type 2 diabetes, who died of kidney cancer, and the paternal grandfather who was affected with coronary artery disease. No further chronic or genetic diseases were reported in other family members. The proband was born to a 36-year-old female with GDM after a 36 week gestation. Delivery was by caesarean section. The mother has had GDM with each of her previous two pregnancies; during this pregnancy, she took subcutaneous insulin twice daily, monitored blood sugar four times per day, and denied worsening of her hyperglycemia during the pregnancy. There was no exposure to alcohol, tobacco, or other teratogenic factors. No additional health problems such as hypertension, obesity, or type 2 diabetes were reported in the mother. Fetal movements were reported as normal. The birth weight was 4.2 kg (>90th centile). The child had a short period of hypoglycemia and received a glucose infusion. He was discharged on the third day of life. On the fourth day of life he was re-admitted due to hyperbilirubinemia and tremors of the upper and lower right limbs. Serum calcium levels were 5.2 mg/dl (normal value: 9–11 mg/dl) and the electrolytes were normal. Therapy with calcium and alpha-calcidiol was successfully started. An echocardiogram showed a patent oval foramen. Cerebral ultrasonography revealed a cyst of the septum pellucidum.

At 24 months, growth and head circumference were normal. Dysmorphic features included long face, a small nose with a normal bridge, long philtrum, highly arched palate, and dental enamel dysplasia (Figure 1a,b). No other craniofacial findings such as, bulbous nasal tip and prominent nasal root, hypoplastic alae nasae, hooded eyelids, cupped and protuberant ears, preauricular pits or tags, or craniosynostosis, which are frequently reported in children with 22q11.2DS, were detected in the patient.

The chest and abdominal examination was normal. A speech delay was observed during the first years of life. At the age of 4 years, expressive language was characterized by a sporadic use of short sentences consisting of 2–3 words, with oculomotor difficulties and abnormalities of perceptual organization. The language impairment was associated with an inhibited temperament and signs of anxiety. However, comprehensive language was appropriate for age. Psychomotor development assessed by Griffith Mental Developmental Scale-Extended Revised (GMDS-ER) was normal (IQ = 86). The patient received psychomotor and speech therapy, resulting in a progressive improvement of the expressive language, even though it remained poorly structured.

Since, the age of 15 months, the patient had recurrent and frequent episodes of URIs. The ear-nose-throat evaluation did not reveal any hearing loss. Ophthalmologic examination was normal. G-banded chromosomal analysis on peripheral blood lymphocytes indicated a normal karyotype.

Fluorescent in situ hybridization performed to exclude a 22q11.2 deletion syndrome and *TBX1* sequencing were both negative.

## 3 | METHODOLOGY AND RESULTS

### 3.1 | Cytogenetic and molecular genetics

A 4 × 44 CytoChip™ array with ISCA design (BlueGnome Ltd; Cambridge, UK) was used in accordance with manufacturer's guidelines for genome screening. The arrays were scanned using the InnoScan 710 and analyzed using BlueFuse for Microarrays 3.5 software (BlueGnome, Cambridge, UK), referring to Hg19 Genome Assembly (NCBI Build GRCh37). Copy number variants were classified according to the Database of Genomic Variants, the DECIPHER Database and the UCSC Genome Browser. Oligo-array CGH analysis identified a 3p12.3 deletion, spanning approximately 317 kb (Figure 2) and overlaps three genes (*LOC401074*, *ZNF717*, *FLJ20518*), *MIR1243*, and *MIR4273* (National Center for Biotechnology Information, hg19). The result, according to the ISCN nomenclature, is: arr [hg19] (75, 571, 183–75, 888, 573 × 1).

The miRBase database available online at http://microrna.sanger.ac.uk/ which provides integrated information about miRNAs and related predicted genes targets, was used to identify the potential genetic targets of the deleted miRNAs. The potential effects of the reduced dosage of the deleted miRNAs on gene targets were considered during the analysis. Among the miRNA-4273 predicted target genes, there are several genes, such as *BMP3*, which are involved in several steps of embryogenesis, such as kidney and lung organogenesis and in insulin gene expression.

### 3.2 | Immunological evaluation

Initial immunological evaluation revealed leukopenia (median white blood cell count ± SD 4900 ± 1237 cells/μl; range over the time

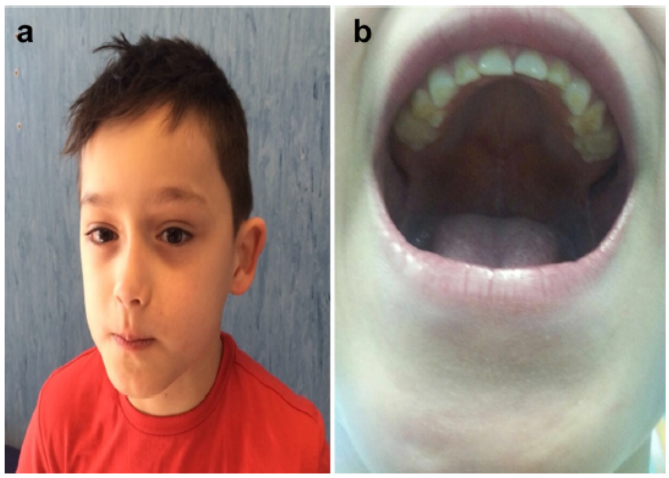


**FIGURE 1** Proband's phenotype. a: Noted long face, long philtrum, and small nose with a normal bridge. b: Highlyarched palate and dental enamel dysplasia. [Colour figure can be viewed at wileyonlinelibrary.com]

3780–7470) with moderate lymphopenia (median 2577 ± 662 cells/μl; range 1170–2670), as illustrated in Table 1. At the diagnosis, the flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) revealed a $T^{low}B+NK+$ combined immunodeficiency (CID), characterized by a marked reduction of CD3+ (373 cell/μl), CD4+ (163 cells/μl), and CD8+ (140 cells/μl) T cells. B lymphocytes were increased (1352 cells/μl), while CD56+ were normal. In order to further investigate the T-cell phenotype and function in the context of a putative thymic a/hypoplasia, we evaluated the naive and memory subsets and the proliferative response to mitogens. As observed in Figure 3, among CD3 + CD4 + T cells, a severe reduction of naïve CD45 RA+ T cells and a prevalence of memory CD45RO + CD4 + T cells were evident. On the contrary, although the number of CD3 + CD8+ T cells was lower than age-matched controls, the distribution of the naïve and memory phenotypes was normal. A reduction of CD4 + CD25+, which include Treg cells, paralleled the CD4+ lymphopenia. The proliferative response to phytohemagglutinin (PHA) and pockweed, evaluated by thymidine uptake from cultured cells pulsed with 0.5 μCi [3H] thymidine (Amersham International), was decreased, corresponding to the 45% and 39% of the control, respectively.

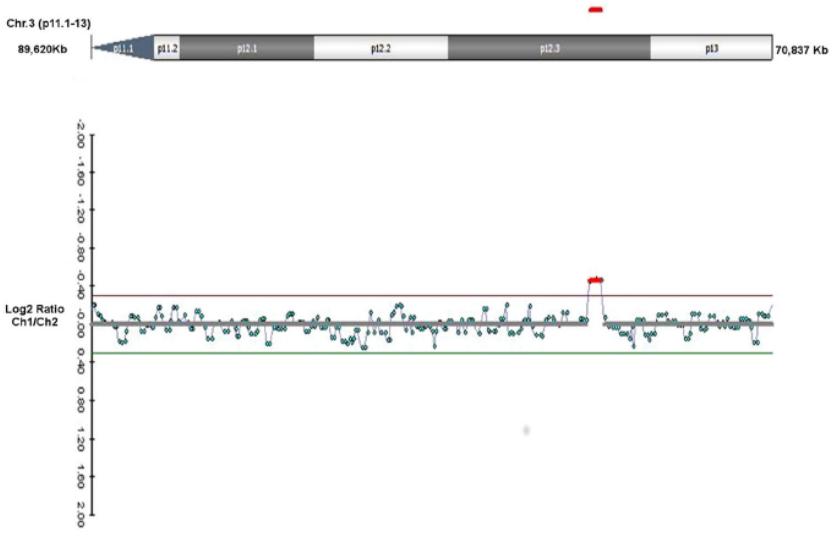


**FIGURE 2** The ideogram of chromosome 3. The red bar indicates the deletion of the 3p12.3 region identified in our patient. [Colour figure can be viewed at wileyonlinelibrary.com]

**TABLE 1** Immunological evaluation during the long term follow-up

| Age (y) | 2 | 2.6 | 3 | 4.6 | 5 | 6 |
|---|---|---|---|---|---|---|
| Serum Ig (mg/dl) | | | | | | |
| IgG | 605 | ND | 809 | 684 | 936 | 800 |
| IgA | 70.9 | ND | 94.3 | 51.7 | 94.7 | 91.6 |
| IgM | 85.4 | ND | 188 | 98 | 130 | 94 |
| Leukocytes (cells/mm$^3$) | 5800 | 4100 | 7470 | 4000 | 4200 | 5160 |
| Lymphocytes (cells/mm$^3$) | 2330 | 1320 | 2670 | 1590 | 1170 | 1370 |
| T cells (CD3+) | 373 (2100–6200) | 396 (1400–3700) | 881 (1400–3700) | 700 (1400–3700) | 526 (1400–3700) | 698 (1400–3700) |
| CD3 + CD4+ | 163 (1300–3400) | 198 (700–2200) | 454 (700–2200) | 318 (700–2200) | 246 (700–2200) | 233 (700–2200) |
| CD3 + CD8+ | 140 (620–2000) | 145 (490–1300) | 267 (490–1300) | 366 (490–1300) | 164 (490–1300) | 192 (490–1300) |
| CD19 | 1351 (720–2600) | 436 (1400–3700) | 854 (1400–3700) | 397 (1400–3700) | 304 (1400–3700) | 110 (1400–3700) |
| CD56 | 419 (180–920) | 396 (130–720) | 667 (130–720) | ND | 316 (130–720) | 507 (130–720) |
| CD4 + CD25 + % | 1 | ND | ND | ND | ND | 1 |
| TCRαβ% | | | | | | 31 |
| TCRγδ% | | | | | | 20 |

The brackets indicate normal values for age; ND, not done.

During the 5 years of follow-up, despite a sporadic mild increase in the lymphocytes count at 3 years of age, a progressive further reduction of lymphocytes was documented.

Even though TCR αβ cells predominated, an increased number of TCR γδ T cells (20%), was detected at the age of 6 years. However, despite the marked reduction of T cells, the patient did not suffer from severe episodes of bacteremia, systemic *C. albicans* infections, or other opportunistic and life-threatening infections. Furthermore, a normalization of the proliferative response to mitogens was observed at 3 years.

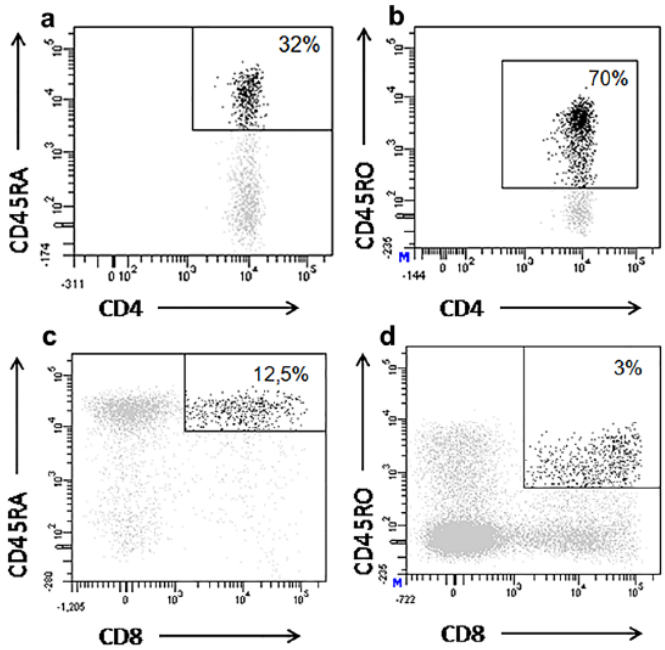


**FIGURE 3** Blood T cell subsets by flow cytometry at the age of two years. a: Naïve T cells (CD45RA +) represent the 32% of the CD4+ cells. b: Memory T cells (CD45RO +) represent the 70% of the CD4+ T cells. c, d: Normal distribution of the naïve and memory CD8+ T cells [Colour figure can be viewed at wileyonlinelibrary.com]

Despite consistently normal serum levels of IgM, IgG IgA, and IgE, the patient had an absent antibody specific production toward Hepatitis B. Autoantibodies to anti-thyroglobulin (TGB-Ab), anti-thyroid peroxidase (TPO-Ab), anti-nuclear (ANA), anti-double stranded DNA (dsDNA), and anti-transglutaminase were all negative.

## 4 | DISCUSSION

In this study, we report a novel association between a DGA characterized by a quantitative and qualitative immunodeficiency resembling a $T^{low}B+NK+$ combined immunodeficiency in a child with a 3p12.3 deletion born to a mother with GDM.

Diabetic embryopathy encompasses a wide spectrum of congenital anomalies with a multifactorial pathogenesis (Castori, 2013). Holoprosencephaly, caudal dysgenesis, VACTERL association, and cardiovascular congenital abnormalities are the most common features associated with diabetic embryopathy. Furthermore, renal dysgenesis has been frequently reported in newborns of diabetic mothers. Moreover, DGA has also been reported in newborns of diabetic mothers (Dentici et al., 2013; Novak, Robert, & Robinson, 2005). In these patients, unilateral o bilateral renal agenesis has also been found, suggesting a non-random association between the two events.

To date, cytogenetic studies in patients with DGA born to diabetic mothers have either not been done or have failed to identify an underlying genetic alteration.

The etiology of diabetic embryopathy is multifactorial and results from the interplay between uterine microenvironment, parental and offspring genomes, and epigenetic regulation (Castori, 2013; Vrachnis et al., 2012). It has been hypothesized that alterations of the metabolic homeostasis associated with DM could profoundly affect several signal transduction pathways and morphogenetic processes (Castori, 2013), thus resulting in developmental field defects. Altered expression of developmental control genes, as *Pax3*, has been reported in mouse models of diabetic embryopathy. However, a molecular mechanism underlying the phenotypic spectrum of DM-associated structural anomalies in humans has not been reported yet. Of note, even though several studies documented a high teratogenic potential for women with pre-gestational DM type 1 (DM1) or 2 (DM2), pregnant women developing GDM have an overall very low risk for fetal congenital anomalies, suggesting the requirement of further pathogenetic cofactors (Balsells, Garcia-Patterson, Gich, & Corcoy, 2012). In our study, we found a novel interstitial deletion at 3p12.3 encompassing three genes, *ZNF717*, *MIR1243* and *MIR4273* in a patient with multiple congenital anomalies born to a mother whose pregnancy was complicated by GDM. MiRNAs are a family of small, non-coding RNAs that modulate gene expression by targeting messenger RNAs for degradation, translational repression or both. MiRNAs may affect a wide range of biological responses including proliferation, differentiation, apoptosis and cell metabolism, and are implicated in several processes, such as brain differentiation and function, growth, and skeletal and cardiovascular development. Individual miRNAs can target multiple messenger RNAs, controlling the expression of several genes, and frequently their alterations can have a profound impact on cellular development and function (Zhang, Wang, & Gameinhart, 2013). Furthermore, there is evidence for miRNA dysregulation and biogenesis having a role in the immunological, cardiac, endocrinological, and neurological phenotype of patients with 22q11.2 DS due to DiGeorge Critical Region Gene 8 (*DGCR8*) haploinsufficiency (de la Morena et al., 2013). *DGCR8* encodes a component of the microprocessor complex involved in miRNA biogenesis, which is deleted in the majority of patients with 22q11.2DS (Sellier et al., 2014). Furthermore, it has been recently demonstrated that the haploinsufficiency of miRNA-17–92 due to a germline hemizygous deletion of *MIR17HG* is responsible for several developmental abnormalities observed in some patients with microcephaly, short stature, and digital abnormalities (De Pontual et al., 2012).

Evidence also indicates that miRNAs may modulate the expression of critical T-box transcriptional regulators during midface development and the BMPs. Part of the *TBX1* mutant phenotype is due to excessive Bmp-signaling (Wang et al., 2013). Interestingly, *BMP3*, a member of the transforming growth factor β superfamily, which plays a key role during embryogenesis, and in particular, in the development of the organs that require an epithelial-mesenchymal interaction (such as the thymus and kidney) is among the miRNA-4273 predicted target genes (Takahashi & Ikeda, 1996). More recently, it has been reported that *BMP3* has a role in the regulation of insulin gene expression in pancreatic beta-cells as well (Bonner et al., 2011). However, a clear causative relationship between haploinsufficiency of *ZNF717* and the patient's clinical phenotype was not found.

In conclusion, we report for the first time on the association between a DGA in an infant born to a mother with GDM and a microdeletion of chromosome 3, involving the *MIR4273* gene. Even though the causal relationship between the two events remains to be proven, our report provides further support for the multifactorial pathogenesis of DGA associated with GDM.

## CONFLICTS OF INTEREST

None declared.

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CME REVIEW

# FOXN1 Deficiency: from the Discovery to Novel Therapeutic Approaches

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**Abstract** Since the discovery of FOXN1 deficiency, the human counterpart of the nude mouse, a growing body of evidence investigating the role of FOXN1 in thymus and skin, has been published. FOXN1 has emerged as fundamental for thymus development, function, and homeostasis, representing the master regulator of thymic epithelial and T cell development. In the skin, it also plays a pivotal role in keratinocytes and hair follicle cell differentiation, although the underlying molecular mechanisms still remain to be fully elucidated. The nude severe combined immunodeficiency phenotype is indeed characterized by the clinical hallmarks of athymia with severe T cell immunodeficiency, congenital alopecia, and nail dystrophy. In this review, we summarize recent discoveries in the field and give interesting perspective about new and promising therapeutic approaches for disorders of immune system with athymia.

**Keywords** FOXN1 · Severe combined immunodeficiency · Athymia · Nude SCID phenotype · Nude mouse · T cell development · Thymus transplantation

## Introduction

Severe combined immunodeficiency (SCID) indicates a clinically and genetically heterogeneous group of congenital disorders due to abnormalities of development and/or function of T, B, and NK cells, always resulting in impairment of both cellular and humoral immunity. To date, more than 20 genetic alterations have been identified as responsible for the disease [1]. Among these, the *FOXN1* gene mutation, causative of the nude SCID phenotype, is the unique condition in which the immunological defect is related to an alteration of the thymic epithelial stroma and not to an intrinsic defect of the hematopoietic cell. The nude SCID phenotype has been identified in human for the first time in 1996 in two female patients who presented with thymus aplasia and ectodermal abnormalities [2], approximately 30 years later than the initial description of the murine counterpart. Thereafter, several nude SCID patients from all over the world have been described in the literature [3–5]. The immunological phenotype is $T^{-/low}B^{+}NK^{+}$, with a profound functional T cell impairment, leading to severe and life-threatening infections in the first months of life. In addition to the classical SCID phenotype, the patients affected also exhibited extra-immunological features, involving primarily the skin and hair.

FOXN1 is a member of the forkhead box gene family that comprises a diverse group of "winged helix" transcription factors implicated in a variety of cellular processes: development, metabolism, cancer, and aging [6]. While during fetal life FOXN1 is expressed in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney, and urinary tract, its postnatal expression is restricted to stromal thymus and skin cells, where FOXN1 is necessarily required for the normal development, function, and maintenance of hair follicles and thymic epithelial cells (TECs). However, the molecular mechanisms by which FOXN1 expression and activity are regulated are only incompletely understood.

The aim of this review is to give an updated and broad picture of the role of FOXN1, and its implications in human disease, based on previously published work, which we hope may be proven useful for both clinicians and scientists in the field. We will begin by summarizing the

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history of FOXN1 discovery in mice and humans, dissecting the human nude SCID phenotype and the disease mechanisms through the elucidation of the role of FOXN1 in the thymus, skin, and nervous system, followed by treatment options and relative outcome of the disease and potential future areas of research.

## The Role of Foxn1 Revealed by Animal Models: the Nude Mouse

The nude mouse phenotype has been described by Flanagan in 1966, after its spontaneous appearance in the Virus Laboratory of Ruchill Hospital, Glasgow, UK [7]. This mouse showed abnormalities of hair keratinization and thymic dysgenesia, resulting in both hairlessness and profound T cell immunodeficiency, indicating that the gene exerted pleiotropic effects. Indeed, positional cloning allowed to identify a member of the forkhead or winged helix superfamily, Foxn1 (originally referred as winged helix nude, *Whn*), as the gene mutated [8–10]. Mice homozygous for the mutation "nude," *nu/nu* mice, were hairless, had delayed growth, decreased fertility, and died early in life because of severe infections. In particular, as concerned the skin, hair follicles were present in the same number in wild-type control and nude mouse; however, in the latter, these follicles result in a hair that started to twist and coil due to the absence of free sulfhydryl groups in the mid-follicle region, thus failing to penetrate into the epidermis. In these mice, moreover, the differentiation of the epidermis was abnormal as well, and characterized by abnormal balance between proliferation and differentiation of keratinocytes in the hair follicle [11, 12]. In addition to these cutaneous abnormalities, the immunological hallmark of the nude mice was an abnormal, or even absent, thymus, resulting in a severe T cell deficiency and an overall severely impaired immune system. The thymus morphogenesis was blocked at the beginning of the development, resulting in a profound alteration of the organ architecture with no subcapsular, cortical, and medullary region formation [9]. In addition, the hair growth could not be rescued by thymus restoration, indicating that the annexa abnormality was due to a direct role of the gene in epidermis differentiation [9, 13]. In support of this, the nude phenotype was also characterized by nail malformations and severe infertility. The first condition was attributed to an abnormal production of fillagrin, a protein of the nail matrix and plate, subsequent to the loss of keratin 1 protein. Differently, the infertility, related to small ovaries with low egg counts in the females and no motile sperm in the males [7], may be the result of hormonal changes, as demonstrated by the altered serum levels of estradiol, progesterone, and thyroxine [14].

## FOXN1 Deficiency in Humans: the Nude SCID

### History

Most of the knowledge on cell-mediated immunity and particularly on T cell thymic lymphopoiesis originated from studies performed in the nude SCID mouse model. Since its first description and the identification of FOXN1 gene, more than 100 papers have been published dissecting its immunological role. As for the human phenotype, for many years, DiGeorge syndrome has been erroneously considered the human counterpart based on the thymic aplasia or hypoplasia [15]. However, DiGeorge syndrome shows a wide spectrum of clinical features, including parathyroid and cardiac and great vessel malformations [16, 17], absent in the nude phenotype, whereas the hallmarks of the nude SCID, hairlessness and abnormalities of skin annexa, are missing. More importantly, the immune defect in DiGeorge patients is also much less severe than the nude phenotype [17]. The identification of the full equivalent human phenotype of nude mouse occurred surprisingly about 30 years later with the description of two Italian sisters, who presented congenital alopecia, eyebrows, eyelashes, and nail dystrophy associated with a severe T cell immunodeficiency, as detailed below [2]. The consanguinity of the parents and the small community where the patients originated from suggested an autosomal recessive inheritance [18]. The time gap from the original mouse description led to hypothesize a lethal phenotype in subjects with complete expression.

### Genetics

FOXN1 gene is located on chromosome 17q11.2 and consists of eight exons, spanning about 30 kb [19] (Fig. 1). Interesting, two different first exons, which are noncoding, have been identified through an extensive screening of cDNA clones obtained from skin cells, the exons 1a and 1b, that undergo to alternative splicing [20]. This suggests the presence of two distinct promoters of exons 1a and 1b [21]. The alternative usage of the exon 1a or 1b seems to be tissue specific, in that promoter 1a is active in thymus and skin, while promoter 1b is active only in skin [20].

Up to date, only three mutations of the *FOXN1* gene have been reported in humans: the R255X, the S188fs, and the R320W [2–5]. These mutations are located in different domains of the molecule, and all resulted in a loss of function of the protein (Fig. 1). The first mutation identified, the R255X, resulted from a homozygous 792C-T aminoacidic transition in the N-terminus exon 5, leading to a premature stop codon causing the truncation of the protein before the start of the evolutionary conserved forkhead domain. The R320W was a homozygous missense mutation located in the forkhead domain (exon 6) in which aminoacidic substitution,

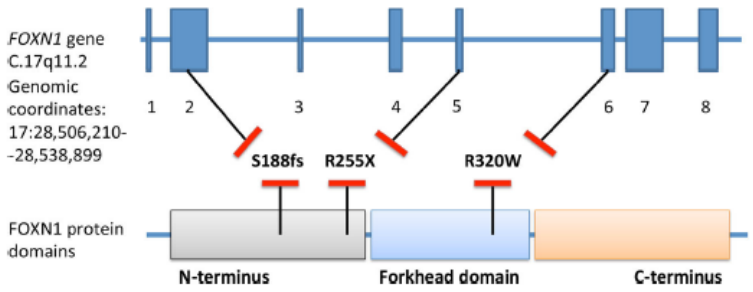


**Fig. 1** Schematic representation of the *FOXN1* gene with the distinct eight exons and protein domains showing the position of identified mutations annotated by amino acid alterations

C987T, impaired its DNA binding ability, and thus the transcriptional regulation of target genes. The last reported S188fs mutation was a small deletion of exon 2, c.562delA, also resulted in a frameshift and premature truncation of the protein after the first 24 amino acids of the forkhead domain.

## Focus on Immunodeficiency: FOXN1 in TECs and T Cell Development

All nude SCID patients reported so far showed decreased T cell counts [2–5], with a predominant reduction of $CD4^+$ T cells [2, 3] and an increase of double-negative lymphocytes in the peripheral blood [3, 4]. T cells had a poor or absent proliferative response to mitogens and exhibited an oligoclonal TCR repertoire [2–4]. NK and B cells, although normal in number, were also functionally impaired with abnormal specific antibody production [2–5]. The T cell immunodeficiency resulted also in a severe reduction of T cell receptor rearrangement excision circles (TRECs) [3], $CD31^+$ recent thymic emigrants [4], and naïve $CD4^+$ $CD45RA^+$ T cells, the latter turning in favor of a $CD45RO^+$ memory phenotype [3, 4, 22].

The identification of the nude/SCID phenotype greatly contributed to unravel important issues of thymic and T cell development. Studies on both mouse and human have demonstrated that the transcription factor FOXN1 plays a key role in the morphogenesis of the three-dimensional thymic architecture, which is important for the functionality of the thymus. In particular, Foxn1 is expressed in all TECs during initial thymus organogenesis and is required to induce both cortical and medullary thymic epithelial cell differentiation [9, 23–26]. Moreover, FOXN1 is considered essential also for the prevention of thymic involution during adulthood [27–29]. In particular, in mice, Foxn1 mainly regulates TEC differentiation and homeostasis during fetal and postnatal life [28, 30]. TECs are implicated in either thymus organogenesis or most stages of the maturation of T cell precursors [31, 32] (Fig. 2). In a Foxn1-dependent manner, TECs release several chemokines, including CCL25, CCL21, and CXCL12, that allow hematopoietic progenitors to enter into the developing thymus [33, 34]. These progenitors, subsequently, are committed to a T cell fate and progress through the different phases of the ontogenesis, thanks to the crosstalk with TECs and under the stimuli of TEC-derived molecules, such as the notch ligand DLL4, which, in turn, is also transcriptionally regulated by FOXN1 [35, 36]. In developing T cells, cortical and medullary TECs (cTECs and mTECs) induce the "positive" and "negative" selection processes, respectively, with mTECs acting only in negative selection [37] (Fig. 2). These processes are driven by MHC–self-antigen complexes presented on the surface of TECs [38, 39] (Fig. 2). In addition to CCL25, CXCL12, and DLL4, FOXN1 has been recently proven to promote the expression of hundreds of genes in TECs that support intrathymic T cell development through the antigen processing and presentation. In particular, it was found that the expression of *Prss16* in cTECs, which encodes a thymus-specific serine protease required for CD4 lineage selection and high MHCII expression, is regulated by Foxn1 [34]. Moreover, a direct binding target of Foxn1 has been detected uniquely in cTECs, represented by a cis-regulatory element involved in the transcriptional promotion of relevant cTEC genes. Indeed, the Foxn1 binding to this element promotes the transcription of β5t gene, the catalytic subunit of cTEC thymoproteasome, which has an essential role in positive selection induction of functionally competent $CD8^+$ T cells within the thymus [40]. In the absence of FOXN1 expression in TECs, the thymic development is thereby blocked at a rudimentary stage [27, 28, 34], characterized by an alymphoid two-dimensional anlage with a cystic structure [22, 41, 42]. In this thymic rudiment, TECs are not capable to allow the hematopoietic precursor cells (HPCs) to enter into the epithelial cluster [43]. Taken together, this evidence strongly demonstrated the pivotal role of the lymphostromal crosstalk. Nevertheless, significant numbers of circulating T cells of non-maternal origin with major expansions of $CD3^+CD4^-CD8^-\alpha\beta$TCR have been documented in all cases reported of homozygous R255X FOXN1 deficiency [2, 3]. Differently, no circulating T cells were found in the patient with the R320W mutation [3]. A possible explanation is that a thymic rudiment may persist, supporting a limited production of T cells. An alternative explanation is that an extrathymic site of T cell differentiation is capable to support the development, albeit with a narrow TCR repertoire and impaired T cell selection. Furthermore, during prenatal T cell

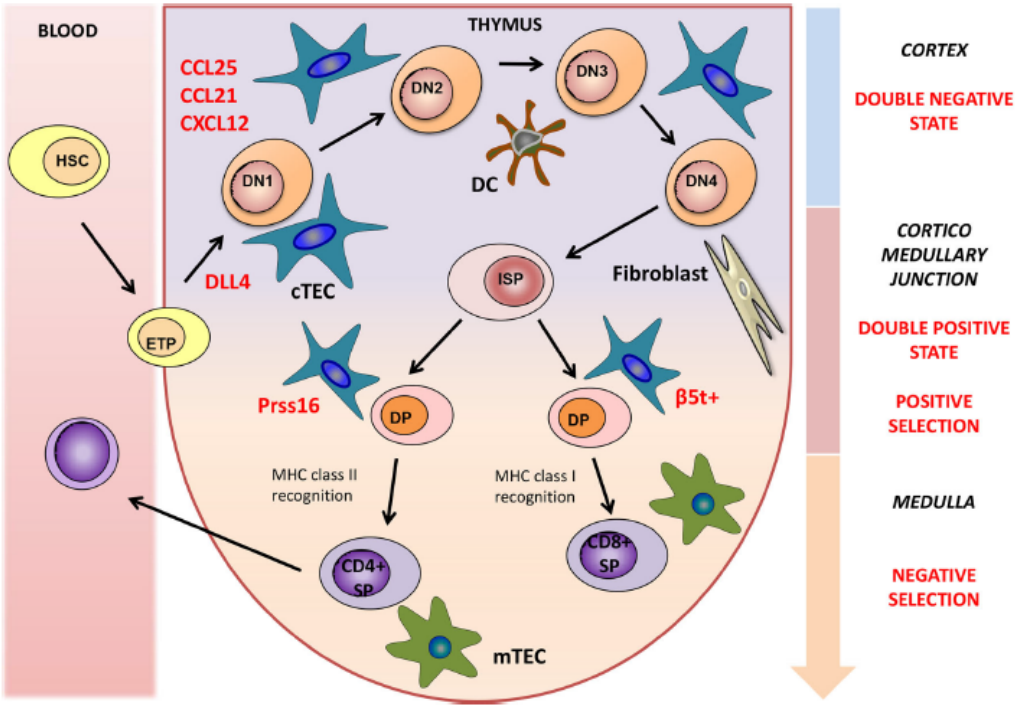


**Fig. 2** Intrathymic T cell development. Lymphoid progenitor cells get access into the thymus through the vessel of the corticomedullary junction under the stimuli of Foxn1-related chemokines released by cTECs. Double-negative (DN) lymphocyte ($CD4^-$ $CD8^-$) interaction with cTECs in the thymic cortex allows them to maturate through different double-negative stages and finally generate double-positive (DP) lymphocytes ($CD3^+CD4^+CD8^+$). cTECs, expressing specific genes under the control of Foxn1, are involved in positive selection. In the medulla, positively selected lymphocytes interact with mTECs to complete the maturation process. Self-reactive lymphocytes are further deleted, through the so-called negative selection. Eventually, single-positive (SP) ($CD3^+CD4^+$ or $CD3^+CD8^+$) lymphocytes are generated and released in the peripheral blood

development in humans, it has been observed that FOXN1 mutation completely abrogates T cell ontogeny of CD4 compartment, while having a limited production of $CD8^+$ cells, indicating a possible different origin of these cells [42].

## Focus on Ectodermal Abnormalities: FOXN1 and the Skin

In addition to athymia and T cell immunodeficiency, FOXN1 mutations are also associated with ectodermal defects of skin and hair, namely alopecia of the scalp, eyebrows, and eyelashes and nail dystrophy. In both patients and nude mice, hair follicles are normal in number but give rise to altered hairs unable to curl and that break off at the level of the skin surface leading to alopecia [7, 44]. As for nail dystrophy, the most frequent features were leukonychia and koilonychia ("spoon nail"), the first one characterized by a proximal arciform alteration of the nail plate and the second one by a concave surface and raised edges of the distal nail plate [45]. Canaliform dystrophy and transverse groove of the nail plate (Beau line) were also noted, although less frequently. The same skin annexa alterations were also reported in a few strains of nude mice [12].

Studies from mouse skin revealed a specific pattern of expression of Foxn1 in both epidermis and hair follicle, indicative of its involvement in cell growth and differentiation processes, and particularly of its role as regulator of starting terminal differentiation [21]. Indeed, in the epidermis, Foxn1 is primarily expressed in the keratinocytes of the first suprabasal layer, which stopped to proliferate and initiated terminal differentiation [12], although rare cell $Foxn1^+Ki67^+$ was found in the basal layer, likely representative of the very early stages of commitment to differentiation [46]. Similarly, in the hair follicle, Foxn1 expression was found in cells located in the supramatrical region and ready to begin terminal differentiation [47, 48]. Functionally, although molecular pathways still remain to be fully elucidated, Foxn1 promotes keratinocyte differentiation through the regulation of more than 50 target genes, including protein kinase B and protein kinase C (PKC), the latter being a potent inhibitor of human hair follicle growth in vitro [49–51]. In keeping with this, PKC activity was found upregulated in $Foxn1^{-/-}$ mouse keratinocytes, while Foxn1 overexpression determined the suppression of PKC activation

and inhibition of keratinocyte differentiation [51]. Studies performed on human epidermal keratinocytes confirmed the role of FOXN1 in the initiation of keratinocyte differentiation, but it was found to be not sufficient to induce the final stages of terminal differentiation [52].

## Focus on Neurological Abnormalities: FOXN1 and the Nervous System

Up to date, central nervous system (CNS) abnormalities have only been found in two fetuses carrying the R255X mutation from the same family in the highly consanguineous village of Acerno [53, 54]. The first fetus showed severe neural tube defects, such as anencephaly and spina bifida, while the second one had milder defects including an enlarged interhemispheric fissure with the absence of the cavum septi pellucidi and corpus callosum [53, 54]. Although other members of the forkhead/winged helix family proteins, such as mouse HNF-3b and BF-1 and human FOXP2, were reported to be involved in CNS development and function [55, 56], the absence of neurological abnormalities in nude mouse models along with the high rate of consanguinity in the population of the two fetuses suggested that another genetic etiology could have caused the neurological features. Thus, the role of FOXN1 in CNS development still remains unconfirmed and to be further investigated [53].

## FOXN1 Mutations in Heterozygous Subjects

In the small community of south Italy, where the first two sisters with FOXN1 deficiency where identified, additional cases of patients with congenital alopecia and early child death because of severe infections were reported [18]. Interestingly, 55 subjects of 843 inhabitants studied were found to carry the heterozygous FOXN1 mutation. All the carriers and affected cases identified belonged to an extended seven generational pedigree, derived by a single ancestral couple born at the beginning of the nineteenth century from which four family groups originated. Physical examination of the identified heterozygous subjects revealed that 39 of the 55 heterozygous subjects showed a nail dystrophy. Leukonychia, characterized by a typical arcifom pattern reminiscent of a half-moon and involving the proximal part of the nail plate, was the most specific phenotypic alteration together with koilonychia and Beau line. Immunological alterations have also been documented in a heterozygous carrier, including lymphopenia and absence of TREC (personal communication by Dr. Gelfand). Unfortunately, no lymphocyte counts or other lab investigations were performed in the large group of heterozygous subjects of south Italy [18].

## Focus on Therapy and Long-Term Outcome: HSCT and Thymus Transplantation

The SCID diagnosis requires a prompt and appropriate treatment, that in the majority of cases is represented by hematopoietic stem cell transplantation (HSCT) that leads to reconstitute the immune system before the onset of life-threatening complications. The sooner the HSCT is performed, the better the outcome [57]. As for nude SCID, since thymic stromal alterations due to FOXN1 mutation underlie the immune defect, thymus transplantation could be a good alternative therapeutic approach to HSCT, even though conclusive results are still not available. Restoring a functional thymic stromal environment is expected to provide a long-lasting immune reconstitution [3, 4]. In complete DiGeorge syndrome, it has been observed that HSCT did not result in a high-quality immune reconstitution [22, 58–61].

Of the nine nude SCID patients reported, five of them have been treated in order to achieve immune reconstitution, three receiving HLA-matched sibling/genoidentical HSCT at 5 months of age [2, 4, 5, 22] and two with thymus transplantation at the age of 9 and 14 months [3]. One of the HSCT recipients 6 years after HSCT was alive and infection-free with reconstitution of $CD3^+$, $CD4^+$, and $CD8^+$ subsets, although naïve $CD4^+$ lymphocyte regeneration and lymphocyte proliferative capacities were impaired [22]. Surprisingly, naïve $CD8^+$ cell was normal, suggesting a different thymus requirement for the generation of naïve $CD4^+$ or $CD8^+$ lymphocytes. The other HSCT recipients died due to post-transplant complications [4, 5]. In the other two cases treated with thymus transplantation, a successful T cell lymphopoiesis was restored with the development of a functional T cell compartment, although it took several months. Both patients showed a normal T cell number, TREC-positive naïve $CD4^+$ T cells, and $CD31^+$ recent thymic emigrants in the peripheral blood. Moreover, the newly generated T cells showed normal proliferative response in vitro, developed a diverse TCR repertoire, and were able to support B cell function, leading to normalization of Ig levels and production of specific antibodies directed against T cell-dependent antigens [3, 62]. The functional immune reconstitution allowed both patients to clear the ongoing pre-transplantation infections and to remain infection-free at 3 and 5 years after thymic transplant, although one patient developed autoimmune hypothyroidism and vitiligo [3, 63].

In conclusion, although thymic transplantation may be considered a promising therapeutic option in nude SCID patients, it should be considered as the most appropriate treatment only if a HLA-matched sibling donor is not available and when a rapid T cell recovery is not needed.

### Future Perspective: Surrogate Organ

Recent evidence documented a new promising perspective, consisting of a scaffold mimicking the three-dimensional architecture of thymus, which may be potentially useful to allow the differentiation of hematopoietic cell precursors and, eventually, the restoration of functional immune system in congenital immunodeficiencies with athymia, such as nude SCID and DiGeorge syndrome. In keeping with this, Clark et al. documented that human skin fibroblasts and keratinocytes arrayed on a synthetic three-dimensional matrix were capable to support the development of functional human T cells from hematopoietic precursor cells in the absence of thymic tissue [64]. The newly generated T cells exhibited the same characteristics of recent thymic emigrants, contained T cell receptor excision circles, possessed a diverse T cell repertoire, and were functionally mature and tolerant to self MHC, indicating a successful differentiation process [64]. Thereafter, using a poly ε-caprolactone (PCL) scaffold reconfiguring a three-dimensional microenvironment, it has been shown that in the absence of thymic cellular epithelial elements, it is possible in succeeding the commitment of lymphoid precursors to the T cell lineage [65–67]. In particular, it has been documented a de novo generation in the matrix of cells of the T lineage expressing surface and molecular markers of early T cell commitment. In particular, a downregulation of *TAL1* and upregulation of *Spi-B* genes, consistently with the loss of the multilineage differentiative potential, was found [65]. Furthermore, *PTCRA* and *RAG2* expressions were also detectable during the culture, indicating that a recombination activity, indispensable for the generation of a T cell repertoire, was active [65]. However, a full maturation process in the matrix was not achieved, suggesting that additional factors or molecular manipulations could be necessary to create a TEC-like surrogate microenvironment capable to support the entire process of T cell ontogenesis. Nevertheless, effort should be maximized in this field of research, since the in vitro re-build of such a surrogate organ capable of reproducing tissue features of primary lymphoid organs is a promising helpful tool for future therapeutic strategies in patients affected with congenital disorders of immune system related to athymia.

## Conclusion

Nude SCID due to FOXN1 mutations is a rare form of immunodeficiency with only a few cases documented up to now, characterized by severe T cell lymphopenia, alopecia, and nail dystrophy. Notably, the nude SCID phenotype is the only form of SCID associated with an alteration of a gene that is not expressed in the hematopoietic cell, but rather related to a peculiar alteration of thymus anlage. This makes the disorder a unique model of disease to investigate molecular pathways involved in thymus dependent and independent T cell ontogeny. However, despite the increasing evidence, the detailed mechanisms of FOXN1 action in thymus and skin still remain to be fully clarified. Additional research in this field would be very helpful in conclusively defining the role of FOXN1 in the biological process and to pave the way for the development of novel therapeutic strategies, such as thymic transplantation or the generation of surrogate organ to treat congenital disorders of immune system.

### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no competing interests.

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## CHAPTER IV

# "New insight on 22q11.2 deletion syndrome"

22q11.2 deletion syndrome (22q11.2DS) is the most common chromosomal microdeletion disorder, estimated to result mainly from de novo non-homologous meiotic recombination events.

The prevalence of the disorder ranges from 1:4000 to 1:6000.

The deletion (approximately 0.7–3 million base pairs in size), results in an heterogeneous clinical presentation, that can be associated with multi-organ dysfunction including cardiac and palatal abnormalities, immune and autoimmune disorders, endocrine, genitourinary and gastrointestinal problems, developmental delays, cognitive deficits and neuropsychiatric illnesses (such as anxiety disorders and schizophrenia) (76, 77).

Palatal abnormalities have been reported in more than half of the subjects. The most common reported ENT disorders is the velopharyngeal incompetence (VPI). The pathogenesis of this condition is multi-factorial.

In the paper published in *J Investigational Allergology Clinical Immunology* we report for the first time a complex pulmonary malformation in a girl affected with 22q11.2DS and recurrent upper and lower respiratory infections. We hypothesize that lung malformations may act as cofactor in the recurrent lower respiratory tract infections in patients with 22q11.2DS

In the second study, submitted on *Pediatric Allergy Immunology* we performed a detailed description of the otolarygological phenotype of a 22q11.2DS cohort, in the attempt to identify functional implications of the anatomical alterations and their possible role in determining susceptibility to infections.

## A Bronchovascular Anomaly in a Patient With 22q11.2 Deletion Syndrome

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**Key words:** 22q11.2 deletion syndrome. Hyperlucent lung. *TBX1*. CT angiography. Recurrent respiratory infections.

**Palabras clave:** Síndrome de deleción 22q11.2. Pulmón hiperlucente. *TBX1*. Angiografía TC. Infecciones respiratorias recurrentes.

DiGeorge syndrome (DGS) is a genetic disorder whose prevalence ranges from 1:4000 to 1:6000 [1]. It is frequently caused by the deletion of a small segment of chromosome 22q11.2 that leads to impaired development of the third and fourth pharyngeal pouches during embryogenesis. In this syndrome, the organs involved include the thymus, parathyroid glands, and heart [2]. The phenotypic spectrum is considerably pleomorphic and includes dysmorphic features, hypocalcemia due to hypoparathyroidism, mild-to-severe immunodeficiency, recurrent infections, feeding and speech difficulties, orthopedic abnormalities, and cardiac defects, such as tetralogy of Fallot, persistent truncus arteriosus, interrupted aortic arch type B, aortic arch anomalies, and atrial or ventricular septal defects [3].

We report the case of a complex pulmonary malformation presenting as hyperlucent lung in a 15-year-old girl with DGS and a history of repeated upper and lower respiratory tract infections requiring monthly antibiotic therapy and chest physiotherapy.

The patient was a second child born by cesarean delivery after an uncomplicated pregnancy to nonconsanguineous white parents. The family history was unremarkable, except for hypertension (father) and chronic obstructive pulmonary disease (maternal grandfather).

At the age of 12 years, she was admitted to the pediatric emergency department because of a new episode of pneumonia associated with hypoxemia. On examination, she had fever, wheezing that was audible in all fields, productive cough, and expectoration. Her facial appearance was unusual and she had cleft palate, hypernasal speech, and dental abnormalities. She had a history of speech delay, even though no mental retardation was documented. Since early childhood, she had experienced recurrent respiratory infections including bronchiolitis and pneumonia.

A diagnostic workup (laboratory and radiological tests) was carried out during admission. Fluorescence in situ hybridization analysis revealed a de novo deletion in region 22q11.2, which is critical for DGS. Color Doppler echocardiography revealed the presence of a patent ductus arteriosus.

In order to exclude the presence of immunological abnormalities associated with DGS, both humoral and cell-mediated immune responses were evaluated. Quantitative serum immunoglobulin levels and the absolute lymphocyte count were within normal limits. Lymphocyte phenotyping, evaluated by flow cytometry, disclosed normal values for $CD3^+$, $CD4^+$, $CD8^+$, and $CD56^+$ cells, whereas $CD4^+CD45RA$ (naive T cells) were slightly reduced, as observed in other DGS patients. Furthermore, the proliferative response to common mitogens was normal.

Two consecutive chest X-ray examinations revealed a band-shaped retrocardiac opacity and hyperlucency of the left lung, both of which persisted following an appropriate course of antibiotics. Advanced diagnostic techniques were requested. Flexible fiberoptic bronchoscopy revealed complete obstruction of the left main stem bronchus in the presence of a pulsation that was synchronous with the heartbeat. Computed tomography (CT) angiography was requested to exclude extrinsic airway compression and revealed a narrowed and virtually collapsed left bronchus with no compressing vascular structure. Subsequent bronchoscopy to confirm the synchronous pulsation was not performed, since it was not strictly indicated for the clinical findings observed. Pulmonary sequestration was ruled out by the absence of an aberrant feeding vessel. In addition, the CT scans revealed hypoplasia of the left pulmonary artery and veins, dilatation of the right pulmonary artery, and enlargement of the left bronchial artery, which was confirmed by subsequent cardiac catheterization. In the lungs, the scan revealed a retracted area of massive consolidation extending from the left hilum to the diaphragmatic pleura and presumably corresponding to a hypoplastic left lower lobe, with evidence of bronchiectasis of the medial-basal segments, along with hyperlucency of the ipsilateral upper lobe and decreased vascularity (Figure). Cardiac magnetic resonance imaging was performed to better clarify bronchovascular morphology and the relationship between the anatomic structures (aortic arch, supra-aortic vessels, ductus arteriosus) and revealed a normal aortic arch, normal pulmonary venous connection with hypoplasia of the left pulmonary veins, dilatation of the pulmonary artery trunk and right main branch, and hypoplasia of the left main branch (maximum diameter 21, 15, and 8 mm, respectively). No evidence of abnormal vessels was found. A small patent ductus arteriosus was evident, even though subsequent echocardiographic monitoring revealed the closure of the ductus. However, the closure did not modify the patient's clinical status.

The radiographic finding of unilateral hyperlucency raised the suspicion of several congenital and acquired diseases [4]. Advanced chest imaging techniques, including bronchoscopy and CT, made it possible to exclude lung parenchymal abnormalities such as bronchial atresia, Swyer-James-MacLeod syndrome, and congenital lobar emphysema.

Hypoplasia of the lung and ipsilateral pulmonary artery are typical features of hypogenetic lung syndrome, also known as scimitar syndrome. Frequently associated with congenital heart diseases such as patent ductus arteriosus and septal defects,

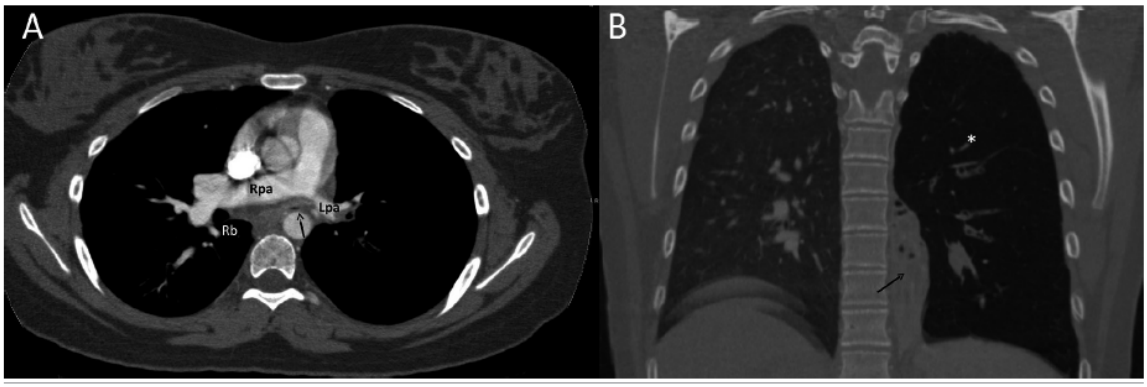


**Figure.** A, Axial multidetector computed tomography image with intravenous contrast (mediastinal window) showing the right bronchus (Rb), the narrowed left bronchus (arrow), hypoplastic left pulmonary artery (Lpa) and hyperplasia of the right pulmonary artery (Rpa). B, Coronal multiplanar reformatted MDCT image (lung window) showing area of consolidation (arrow) and hyperlucency (asterisk) in the left lung.

the syndrome presents with exertional dyspnea and recurrent respiratory infections. It usually affects the right side and is accompanied by dextrocardia and anomalous pulmonary venous drainage and systemic arterial blood supply [5], none of which were encountered in our case.

Both tracheobronchial and pulmonary malformations have been reported as part of DGS and may influence its natural history and surgical treatment [6,7]. Bertolani et al [8] hypothesized a causal connection due to a defective mesenchymal–epithelial interaction, supported by evidence of the migration of concomitant neural crest cells and budding of the tracheobronchial tree, during the fourth week. This may explain the bronchial obstruction observed in the present case.

As for the remaining anomalies described, *TBX1* haploinsufficiency, which is implicated in shaping the DGS phenotype, is thought to play a pivotal role in vascular anomalies. This gene encodes for a transcription factor whose downstream targets are involved in the migration of neuroepithelium-derived cardiac neural crest cells, a process that is, in turn, essential for the development of the aorta and pulmonary trunk from the cardiac outflow tract [9]. Findings from recent studies indicate that *TBX1* also coordinates angiogenesis in the brain by regulating *VEGFR3* and *DLL4* in endothelial cells, thus leading to vascular defects in the brain of knockout mice [10].

In conclusion, we report the first case of a complex malformation including narrowed main stem bronchus and hypoplastic lung and pulmonary artery in a patient with DGS. The malformation was presumably directly or indirectly related to alteration of *TBX1*. We hypothesize that this malformation is a cofactor in the recurrent lower respiratory tract infections affecting this patient. Therefore, its presence should be considered in patients affected with 22q11.2 deletion syndrome.

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*Conflicts of Interest*

The authors declare that they have no conflicts of interest.

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# Otolaryngological features in a cohort of 22q11.2 deletion syndrome patients: a monocentric survey.

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**Title:** Otolaryngological features in a cohort of 22q11.2 deletion syndrome patients: a monocentric survey.

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**Abstract (244 words)**

**Background:** Otorhinolaryngologic manifestations are common in 22q11.2 deletion syndrome (22q11.2DS), but poorly described. This study aimed to better define the ear-nose-throat (ENT) phenotype of 22q11.2DS patients, in the attempt to best detect subjects requiring subspecialist intervention.

**Methods:** We enrolled 25 patients affected with 22q11.2DS. Anatomic and functional ENT findings were investigated using clinical, laboratory and instrumental data. Immunophenotype and frequency of infections were evaluated. Univariate and multivariate analyses were performed.

**Results:** ENT anomalies were found in 88% of patients, and in 20% congenital palate defects required surgery. Adenoids hypertrophy or palatine tonsils hypertrophy were noted in 80 and 48%. Fifty-two percent of subjects had rhinolalia/phonia, severe in half of these. We also found nasal regurgitation or laryngeal penetration/aspiration in 20 and 16%, respectively. Instrumental exams revealed a mild conductive hypoacusia in 32% (bilateral in most cases), tympanometric anomalies in 28%, and swallowing abnormalities in 16%. Statistical univariate analysis showed a direct association between rhinolalia/phonia and episodes of laryngeal aspiration (P=0.016) and between tympanometric anomalies and

increased adenoid volume (P=0.044). No association between episodes of food aspiration and palatal anomalies was found. Moreover, no statistically significant association was observed between the number of ENT district infections and the ENT findings.

**Discussion:** This study contributes to better define the ENT phenotype in patients with 22q11.2DS, helpful to prevent potential complications. Furthermore, the identification of a subcategory of patients may allow the early adoption of specific speech therapy programs to improve the clinical outcome of 22q11.2DS patients.

**Key words:** 22q11.2 deletion syndrome, DiGeorge Syndrome, ENT phenotype, Palatal defects, Primary Immunodeficiency, Recurrent respiratory infections

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## Introduction

Chromosome 22q11.2 deletion syndrome (22q11.2DS), also called DiGeorge or velocardiofacial syndrome, occurs in approximately 1:4000 live births (1, 2). The phenotype of this syndrome is complex and the clinical expression is highly variable. Major clinical features include conotruncal cardiac defects, neonatal hypocalcemia due to hypoparathyroidism, immunodeficiency due to thymic a/hypoplasia, recurrent respiratory infections, facial anomalies, intellectual disability and speech delay. Renal, skeletal and gastrointestinal anomalies may also be observed (3-8). Psychiatric or autoimmune disorders may also be part of the clinical phenotype, in particular in older subjects (9). The 22q11.2DS also includes ear-nose-throat (ENT) manifestations, whose variable expressivity has been poorly characterized so far.

Palatal abnormalities have been reported in more than half of the subjects, and the most common of these is velopharyngeal incompetence (VPI). The pathogenesis of this condition is multi-factorial, being linked to an anatomical problem (short palate), a functional problem (musculature hypotonia), or a combination of both. Some patients exhibit submucosal cleft palate or bifid uvula, whereas overt cleft palate and cleft lip/palate are less common. Furthermore, most 22q11DS patients have speech or language difficulties related to velopharyngeal and palatal defects, which may present as hypernasal speech or increased nasal resonance (10, 11). Conductive bilateral hearing loss is frequently reported, especially in subjects with recurrent acute bacterial or secretory otitis media. It is hypothesized that the anatomical and functional defects of palate and of Eustachian tube, in association with the immune deficiency, may contribute to the conductive loss. Only the 15% suffered of sensorineural deafness (9, 12-14).

to enable a clearer interpretation of the results. Variables showing distribution to be highly skewed were analyzed on the log scale. The calculations were performed using IBM SPSS Statistics, v.20.0 software (IBM Corp. Armonk, NY, USA).

**Results**

Our cohort of patients, as illustrated in Table 1, included 25 subjects (16 males), aged 4-21. All patients had a partial 22q11.2DS phenotype and approximately 76% of them had a cardiac cono-truncal defect. Eighty-eight percent of the patients had ENT abnormalities and 20% required surgical intervention for congenital palatal defects

The patients have been investigated about a number of anatomical and functional otolaryngological features, as detailed in Table 2. Twenty percent of the subjects had congenital major palatal abnormalities, including submucous cleft palate, cleft velum and cleft palate. Minor palatal abnormalities affected 21 of the 25 patients (84%): more than half of them (56%) had occult submucous cleft palate, while 20% of the subjects had a hypomobile palate, and 8% had an immobile palate. The otological exam showed normal tympanic membranes in 16 of the 25 patients (64%), bilateral retracted tympanic membrane in 8 of 25 (32%), and bilateral tympanic effusion in 1 subject (3.8%). As for the palatine tonsils volume, 13 of the 25 patients (52%) had tonsils not exceeding the pillars, 10 patients (40%) had tonsils up to 50% of the space between loggia and median line, and 2 patients (8%) had tonsils up to 75%. Moreover, 5 of the 25 patients (20%) had adenoids occupying up to 25% of the nasopharynx, 15 (60%) had adenoids occupying up to 50% and 5 (20%) had adenoids occupying up to 75% of the nasopharynx. As for the presence of rhinolalia/rhinophonia, 13 subjects (52%) had normal articulation, 7 (28%) had some typical slight articulatory defects with mild/moderate open rhinophonia (hypernasality), and 5 (20%) had constant articulatory defects with moderate/severe open rhinophonia. About the study of nasal air emission, audible inconsistent nasal emission in 7 cases (28%) and audible consistent nasal emission in 6 out of 25 cases (24%) was highlighted. Episodes of laryngeal aspiration/penetration were reported in 4 out of 25 subjects (16%), while

episodes of nasal regurgitation in 7 patients (28% of the cases). Concerning the presence of food consistency restrictions, we found restriction in 1 food consistency (solid) in 5 of 25 subjects (20%), and in 2 or 3 consistencies in 2 (8%). Finally, about respiratory patter in our patients, 5 out of 25 subjects (20%) were mouth breathers and 8 out of 25 (32%) were mixed breathers.

Instrumental investigations were carried out to better characterize the patients. Bilateral type B tympanogram was found in 5 out of 25 subjects (20%), unilateral type B in 1 (4%), bilateral type C in 1 (4%), and bilateral type A tympanogram in the remaining 18 patients (72%). Audiometry revealed mild bilateral conductive hearing loss (25-40 dB) in 7 patients (28%), mild unilateral conductive hearing loss (25-40 dB) in 1 (4%), bilateral normoacusia in 17 patients (68%). Videofluoroscopy was normal in 21 patients (84%); the exam showed hypopharyngeal residues after swallowing in 2 cases (8%), and nasal regurgitation with or without hypopharyngeal residues after swallowing in other 2 cases (8%).

Statistical analysis to identify associations between the anatomical, functional as well as instrumental alterations of otolaryngologic phenotype is illustrated in Tables 3 and 4. As illustrated in Table 3, a significant association between the severity of the major palatal anomalies at birth, requiring surgery, and the degree of rhinolalia/phonia ($P=0.017$), as well as between major palatal anomalies and nasal air emission ($P=0.012$) was found. As expected, a correlation was also found when minor palatal abnormalities were compared with the degree of rhinolalia/phonia ($P<0.0005$) and with nasal air emission ($P<0.0005$). Rhinolalia/phonia grading also correlated with the presence of episodes of laryngeal aspiration/penetration ($P=0.016$) and with severity of food restriction ($P=0.001$). There was also a correlation between nasal regurgitation and laryngeal

aspiration/penetration (P=0.009). In addition, food restrictions were significantly associated with nasal air emission (P=0.007), with nasal regurgitation episodes (P<0.0005), with the presence of episodes of laryngeal penetration/aspiration (P<0.0005), and with mouth breathing pattern (P=0.019). As illustrated in Table 4, the tympanometric pattern correlated, as expected, with the abnormal otoscopic findings (P=0.022) and with the adenoid volume (P=0.044); there was also the association with mouth respiratory pattern (P=0.020). Similarly, the more severe audiometric anomalies the more profound abnormal respiratory pattern (P= 0.002) was noted. Eventually, the abnormal transit of the bolus, detected at videofluoroscopy, correlated with episodes of laryngeal aspiration/penetration (P=0.016), with a linear progression.

We also studied the immunophenotype in our cohort of subjects. Lymphopenia affected 4 of 25 patients (16%). Nine of 25 patients (36%) had a reduced number of CD4+ cells compared to normal range for age. Eleven of 25 (44%) and 4 of 25 (16%) patients had a reduced number of CD8+ or CD19+ cells, respectively. About serum levels of immunoglobulin of our 25 patients, 1 (4%) had a reduced serum concentration of IgA, 8 (32%) had reduced levels of IgM, and 1 (4%) patient had reduced concentration of IgG compared to normal range for age.

The mean (± s.d.) of the number of respiratory infections in the patients group during the last 24 months was 2.77 ± 2.96; 6 of them had a number of infections > 6. No statistically significant association was observed between the number of ENT district infections and the ENT findings already mentioned. Moreover, there was no association between infection and immunophenotypic findings.

As shown in the Fig 1, using the Pearson correlation test we observed a direct correlation between lymphocyte cells count and the severity of audiometric alteration (P=0.007) and the palatine tonsils volume (P=0.013).

We next performed multivariate analysis (namely factor analysis) to obtain information about the interdependency between the observed variables in our cohort of 22q11.2DS patients. As detailed in the Fig 2, we found 6 clusters of variables (defined as components), where a few variables are correlated one to each other. In particular, as illustrated in the Figure 2, Panel B), 4 of them are represented by functional and anatomical ENT features. The first ENT cluster includes variables related to swallowing disturbances, namely food restriction, episodes of nasal regurgitation or laryngeal penetration/aspiration; the second one includes variables related to speech disturbance, such as nasal air emission, rhinolalia/phonia grading and minor palatal abnormalities; the third and the fourth group concern otological and upper respiratory tract disturbances, being represented by tympanometric alterations and severity of conductive hearing loss or by adenoid volume and mouth breathing pattern, respectively. Further 2 clusters refer to immunological parameters.

early detecting bolus transit abnormalities. This could also pave the way to set up strategies to prevent associated complications, as *ab ingestis* pneumonitis. Furthermore, the early adoption of a specific speech therapy program could be helpful in improving the overall quality of life of such patients (21).

Any association was observed between episodes of nasal regurgitation and palate anomalies (including major and minor abnormalities), nor between the latter and episodes of laryngeal penetration/aspiration, differently from what observed in patients not affected with 22q11.2DS with palate abnormalities, in whom a positive correlation was found (22). This finding would suggest that further factors, as dysmorphic features or muscular dysmotility, may be implicated in the pathogenesis of dysphagia and nasal regurgitation associated with 22q11.2DS.

The instrumental study of the acoustic function was performed by audiometry and timpanometry. The results appear to be substantially in line with those described in larger studies (14, 23). Statistical analysis showed that abnormal timpanometric findings were directly related to the audiometric and otoscopic alterations, and with the adenoid volume, as well. These data suggest that a careful management of adenoid hypertrophy should be considered to prevent tympanometric alterations in patients with 22q11.2DS. Regarding the respiratory pattern, there was an interdependence between mouth breathing and increased adenoid volume. This observation would favor the hypothesis that adenoid hypertrophy could play a causal role in determining respiratory pattern of patients with 22q11.2DS, differently from what previously thought on the role of neurological status or narrow nasal vault in causing mouth breathing pattern (10).

Eventually, although we couldn't find any association between the ENT findings and the number of infections of the ENT district, we cannot conclusively rule out that ENT

abnormalities may have a role in favoring infections due to the limited number of patients studied. A multicentric study, enrolling a larger cohort of patients, could be helpful in answering to this issue.

**Legend to the figures**

**Figure 1** Univariate analysis. Box plots show the distribution of the variable "Lymphocyte cells count" in our cohort of patients, using like grouping variables "Audiometry" and "Palatine tonsils volume". The analyses suggest positive association between "Lymphocyte count" and the two variables (P=0.007, P=0.013 respectively).

**Figure 2** Multivariate analysis. All components with eigenvalues under 1.5 were dropped. Varimax rotation method was used. Factor loadings in multivariate analysis, including all the variables is illustrated in the Panel B). Factor analysis shows that within each factor there are clusters of variables, which are positively correlated one to each other.

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**Table 1** Main descriptive data of the cohort of 22q11.2DS patients

| N of patients | 25 |
|---|---|
| Age, *years* mean (SD) | 10.6 (2.77) |
| Male (%) | 64 |
| Partial Del22 phenotype (%) | 100 |
| ENT anomalies (%) | 88 |
| ENT surgery (%) | 20 |
| Logopedic therapy (%) | 60 |
| Congenital cardiac defects (%) | 76 |
| Hypoparathyroidism (%) | 12 |

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| Hypoparathyroidism (%) | 12 |

**Table 2** Frequency of ENT features in the cohort of patients

| | n | % |
|---|---|---|
| Previous surgery | | |
| no surgery | 20 | 80 |
| submucous cleft palate | 2 | 8 |
| cleft velum | 2 | 8 |
| cleft palate | 1 | 4 |
| Palatal classification | | |
| no anatomical and functional anomalies | 4 | 16 |
| minimal anomalies (occult submucous cleft) | 14 | 56 |
| minor anomalies with palatal hypomotility | 5 | 20 |
| major anomalies with absent motility | 2 | 8 |
| Nasal air emission | | |
| no air emission on pressure sensitive sounds | 12 | 48 |
| audible inconsistent nasal emission | 7 | 28 |
| audible consistent nasal emission | 6 | 24 |
| inaudible nasal emission | 0 | 0 |
| Rhinolalia/rhinophonia classification | | |
| normal articulation without appreciable alterations of resonance | 12 | 48 |
| some typical slight articulatory defects with mild/moderate open rhinophonia | 7 | 28 |
| typical and consistent articulatory defects with moderate/severe open rhinophonia | 5 | 20 |
| Palatine tonsils volume | | |
| tonsils not exceeding the pillars (0%) | 13 | 52 |
| tonsils up to 50% of the space between loggia and median line | 10 | 40 |
| tonsils up to 75% | 2 | 8 |
| tonsils up to 100% | 0 | 0 |
| Adenoids volume | | |
| adenoids occupying up to 25% of the nasopharynx | 5 | 20 |
| adenoids occupying up to 50% | 15 | 60 |
| adenoids occupying up to 75% | 5 | 20 |
| subtotal or total obstruction of nasopharynx | 0 | 0 |
| Otoscopic findings | | |
| normal tympanic membrane on both sides | 16 | 64 |
| bilateral retracted tympanic membrane | 8 | 32 |
| bilateral tympanic effusion | 1 | 4 |
| Tympanogram | | |
| type A bilaterally | 18 | 72 |
| type C bilaterally | 1 | 4 |
| type A on one side and type B on the other | 1 | 4 |
| type B bilaterally | 5 | 20 |
| Hearing | | |
| normal hearing (<25 dB) | 17 | 68 |
| mild monolateral conductive hypoacusia (25-40 dB) | 1 | 4 |
| mild bilateral conductive hypoacusia (25-40 dB) | 7 | 28 |
| moderate conductive hypoacusia (40-60 dB) | 0 | 0 |
| Episodes of laryngeal aspiration/penetration | | |
| no current or previous disorder | 21 | 84 |
| previous episodic disorders | 3 | 12 |
| previous and current episodic disorders | 1 | 4 |
| Episodes of nasal regurgitation | | |
| no current or previous disorder | 18 | 72 |
| previous episodic disorders | 4 | 8 |
| previous and current episodic disorders | 3 | 12 |
| Swallowing anomalies | | |
| no abnormalities | 21 | 84 |

| | | |
|---|---|---|
| hypopharyngeal residues after swallowing | 2 | 8 |
| nasal regurgitation with or without hypopharyngeal residues | 2 | 8 |
| Respiratory pattern | | |
| nasal | 12 | 48 |
| mixed | 8 | 32 |
| oral | 5 | 20 |
| Food restrictions | | |
| no restrictions | 18 | 72 |
| in 1 consistency | 5 | 20 |
| in 2 or 3 consistencies | 2 | 8 |

n: absolute number of patients per feature

**Table 3** Univariate analysis. Relationships between anatomic and functional variables in our cohort of subjects

| | Rhinolalia/phonia grading | Nasal air emission | Episodes of nasal regurgitation |
|---|---|---|---|
| Surgery | | | |
| Spearman's rho | 0.472 | 0.494 | 0.117 |
| p-value | **0.017** | **0.012** | 0.578 |
| Minor palatal abnormalities | | | |
| Spearman's rho | 0.754 | 0.746 | 0.288 |
| p-value | **0.000** | **0.000** | 0.163 |
| Episodes of laryngeal P/A* | | | |
| Spearman's rho | 0.476 | 0.328 | 0.511 |
| p-value | **0.016** | 0.110 | **0.009** |
| Food restrictions | | | |
| Spearman's rho | 0.604 | 0.522 | 0.680 |
| p-value | **0.001** | **0.007** | **0.000** |

*P/A: penetration/aspiration

**Table 4** Univariate analysis. Relationships between instrumental ENT variables and anatomic-functional ENT variables in our cohort of subjects

| | Otoscopic anomalies | Adenoid volume | Respiratory pattern | Episodes of laryngeal P/A* |
|---|---|---|---|---|
| Tympanometric anomalies | | | | |
| Spearman's rho | 0.457 | 0.407 | 0.453 | -0.020 |
| p-value | **0.022** | **0.044** | **0.023** | 0.925 |
| Audiometric alteration | | | | |
| Spearman's rho | 0.382 | 0.280 | 0.571 | 0.204 |
| p-value | 0.059 | 0.176 | **0.003** | 0.327 |
| Videofluoroscopic pattern | | | | |
| Spearman's rho | 0.073 | 0.000 | 0.078 | 0.475 |
| p-value | 0.728 | 1.000 | 0.709 | **0.016** |

*P/A: penetration/aspiration

Fig 1

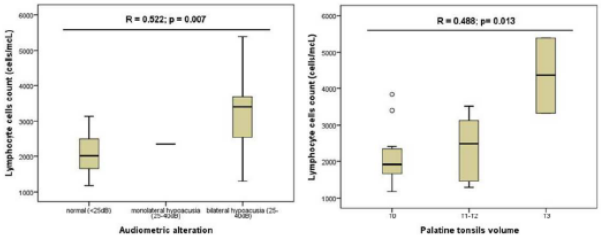


Figure 1 Univariate analysis. Box plots show the distribution of the variable "Lymphocyte cells count" in our cohort of patients, using like grouping variables "Audiometry" and "Palatine tonsils volume". The analyses suggest positive association between "Lymphocyte count" and the two variables (P=0.007, P=0.013 respectively).

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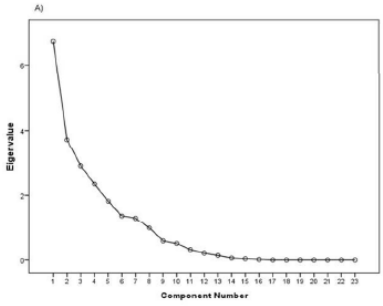
Fig 2




B)

| | Component | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| CD3+ cells count | ,940 | | | | | |
| Lymphocyte cells count | ,920 | | | | | |
| CD4+ cells count | ,905 | | | | | |
| CD19+ cells count | ,806 | | | | | |
| CD8+ cells count | ,792 | | | | | |
| IgA serum level | | | | | | |
| Food restrictions | | ,842 | | | | |
| Episodes of nasal regurgitation | | ,839 | | | | |
| Episodes of laryngeal penetration/aspiration | | ,774 | | | | |
| Videofluoroscopy | | | | | | |
| Minor palatal abnormalities | | | ,856 | | | |
| Nasal air emission | | | ,836 | | | |
| Rhinolalia/phonia grading | | | ,800 | | | |
| Major palatal abnormalities | | | | | | |
| IgG serum level | | | | ,926 | | |
| Total Ig serum level | | | | ,927 | | |
| Tympanometry | | | | | ,820 | |
| Hearing | | | | | ,818 | |
| Otoscopic findings | | | | | | |
| Adenoid volume | | | | | | ,862 |
| Respiratory pattern | | | | | | ,772 |
| Palatine tonsils volume | | | | | | |
| IgM serum level | | | | | | |

Figure 2 Multivariate analysis. All components with eigenvalues under 1.5 were dropped. Varimax rotation method was used. Factor loadings in multivariate analysis, including all the variables is illustrated in the Panel B). Factor analysis shows that within each factor there are clusters of variables, which are positively correlated one to each other.

338x190mm (96 x 96 DPI)

# CHAPTER V

# “Primary immunodeficiency with ectodermal disorders”

Skin and skin annex abnormalities may be a warning sign of immunodeficiency, since both epidermal and thymic epithelium have ectodermal origin.

Recent evidence highlights that skin participates in the host defenses either acting as a primary boundary for germs, as the principal site of environment–host interactions, or directly in the developmental process of the immune system. As matter of fact, skin and skin annex abnormalities, as skin dryness, brittleness of hair, nail abnormalities and abnormal dentition, can be frequently associated with distinct forms of PIDs (78).

FOXN1 is a developmentally regulated transcription factor, selectively expressed in epithelial cells of the skin and thymus, where it plays a necessary role for T lymphopoiesis by inducing a proper epithelial cell differentiation and endothelial cell/thymic mesenchyme communication network (79). FOXN1 deficiency prevent the development of the T-cell compartment, associated to ectodermal abnormalities, such as alopecia and nail dystrophy (80-83).

Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), formerly known as autoimmune polyendocrine syndrome type 1, is a paradigm of a monogenic autoimmune disease caused by mutations of autoimmune regulator (*AIRE*) gene. AIRE acts as a transcription regulator that promotes immunological central tolerance by inducing the ectopic thymic expression of many tissue-specific antigens. Although the syndrome is a monogenic disease, it is characterized by a wide variability of the clinical expression with no significant correlation between genotype and phenotype. Indeed, many aspects regarding the exact role of AIRE and APECED pathogenesis still remain unraveled (84, 85).

In the Brief communication published in *Clinical Immunology* we investigated the pathogenesis of humoral alterations in patients with Hypoidrotic ectodermal dysplasia due to mutations in the NK-kB essential modulator (NEMO) and in a patient with mutations in ectodysplasin A (EDA**).**

In the following 2 reviews published in *International Reviews of Immunology* and *Frontiers in Pediatrics*, we described the link between ectodermal disorders and PIDs and summarized recent novelties on molecular mechanisms underlying the development of APECED and their clinical implications.

A case report describing new phenotypic findings in a patient affected with Incontinentia Pigmenti has been submitted on *British Journal of Dermatology*.

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Brief Communication

# B cells from nuclear factor kB essential modulator deficient patients fail to differentiate to antibody secreting cells in response to TLR9 ligand

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ABSTRACT

Hypohidrotic ectodermal dysplasia (HED) consists of disorders resulting from molecular alterations of ectodysplasin-A (EDA) pathway. Hypomorphic mutations in NF-kB essential modulator, downstream EDA, result in HED with immunodeficiency (HED-ID), characterized by susceptibility to encapsulated pyogenic bacteria infections. Increased susceptibility to pneumococcal infections and poor response to polysaccharide antigens are associated with defect in T-independent B-cell immunity. We investigated B-cell differentiation and immunoglobulin secretion induced by the TLR9 ligand CpG in two HED-ID and in a HED patient caused by *EDA* mutations (XLHED). In HED-ID, only few B cells differentiated into plasma cells upon TLR9 stimulation and memory B cells did not produce IgG and IgA, but small amounts of IgM. Unexpectedly, memory B cells from XLHED patient failed to produce normal IgA or IgG amount upon TLR9 stimulation. Our findings expand the knowledge about the pathogenesis of humoral alterations in HED patients and help explain the susceptibility to pneumococcal infections.


## 1. Introduction

Hypohidrotic ectodermal dysplasia (HED) is a group of rare inherited disorders that affect tissues of ectodermal origin with an incidence of seven cases per 10,000 live births [1,2]. HED derives from mutations in the ectodysplasin-A (EDA) signaling pathway, which leads to the expression of genes implicated in the development of the skin and skin appendage. Mutations in the *EDA* gene on X-chromosome cause approximately 80% of cases of HED (OMIM 305100, XLHED, ectodermal dysplasia, type 1, ED1). A smaller subset of cases is caused by mutations in the EDA receptor (EDAR), the adapter protein (EDARADD), or WNT10A [3], being inherited in an autosomal recessive (ectodermal dysplasia anhidrotic; EDA; OMIM 224900) or autosomal dominant manner (ectodermal dysplasia type 3; ED3; OMIM129490). Hypomorphic mutations in the NF-kB essential modulator (NEMO) encoded by the *IKBKG/NEMO* gene on the X-chromosome, result in HED with immunodeficiency (HED-ID, OMIM 300291) [4–6]. HED-ID has estimated incidence of 1:250,000 live male births [7]. Due to the pleiotropic role of NEMO, mutations in *IKBKG/NEMO* gene lead to heterogeneous and severe immunodeficiency characterized by hypogammaglobulinemia, defect in the antibody response to polysaccharide and proteic antigens and elevated class M immunoglobulin (Ig) levels [8,9]. Immunodeficiency results in susceptibility to encapsulated pyogenic bacteria, such as *Streptococcus pneumoniae*and *Haemophilus influenzae*, mycobacteria, and herpes virus infections [10,11]. Even though NEMO has been shown to be involved in different pathways, thus explaining the complexity of the immunological phenotype, little is known about the pathogenesis of the humoral defects. The involvement of NF-kB in the CD40 signaling pathway may explain only in part the humoral alterations. In fact, in a large cohort of patients, hypogammaglobulinemia occurred in 59% of the patients, but only in Zinc Finger mutations it was correlated with impaired CD40 signaling. Growing body of evidence demonstrates the role ofT-independent B-cell immunity in the response against polysaccharides of encapsulated bacteria [12]. Recurrent lower respiratory tract infections caused by encapsulated bacteria might cause permanent organ damage in patients with common variable immunodeficiency (CVID). Despite the profound hypogammaglobulinemia, some patients do not experience bacterial pneumonia. Studies suggest that alterations of IgM memory B cells, T-independent B cell differentiation and the ability to generate anti-pneumococcal polysaccharide IgM [13] discriminate patients at higher risk of recurrent infections caused by encapsulated bacteria [14], similarly to what happens in splenectomized or asplenic patients [12]. Recent evidence indicates

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that the differentiation of transitional B cells into IgM memory B cells requires a proper Toll-like receptor 9 (TLR9) signaling [15]. Although TLR9 signaling can activate memory B cells directly, additional signals, like RP105, seem to be required for efficient naïve B cell responses [16]. Intriguingly, patients with IRAK-4 and Myd88 deficiencies, which are implicated in Toll–IL-1R (TIR) signaling pathway, acting upstream NEMO, are also highly susceptible to invasive bacterial infections caused by *S. pneumoniae*[17]. Recent studies show that patients affected with IRAK-4 and Myd88 deficiencies have fewer IgM + IgD + CD27 + B cells, reduced serum IgM antibody recognizing T-independent bacterial antigens, and impaired TLR-induced proliferation of IgM + IgD + CD27 + B cells in vitro [18]. This evidence could suggest that also in HED-ID patients, bacterial diseases may be due, at least in part, to the impact of NEMO mutations on the TIR signaling pathway. To date, little is known about the role of T-independent B-cell immunity in susceptibility to infections from encapsulated bacteria in HED-ID. In this study we investigated B-cell differentiation and Ig secretion induced by the TLR9 specific ligand CpG in HED-ID patients. We also studied a patient with HED due to mutations in the *EDA* gene on the X-chromosome (XLHED), which is not implicated in TIR signaling pathway.

## 2. Methods

### 2.1. Patients

Patients herein reported are in follow-up at the Federico II University. For each patient, routine examination, serum Ig concentrations andleukocyte counts were evaluated through standard methods and compared with laboratory-specific age-related normal values. All studies were performed with informed parental consent.

### 2.2. IKBKG and EDA-1analyses

Genomic DNA was prepared by means of phenol–chloroform extraction, RNA by means of Trizol reagent (Invitrogen, Carlsbad, California), and cDNA by means of Superscript reverse transcriptase PCR system (Invitrogen), all according to manufacturer recommendations.

*IKBKG*-specific primers were used to evaluate the full cDNA with the following primer sets, as previously described [19]: forward, 5#-CCCTTGCCCTGTTGGATGAATAGGC-3#; reverse, 5#-AGGCGGAGAGGAAAGCGCAGACTG-3#; and forward, 5#-AAGCTGGCCCAGTTGCAGGTGGCCT-3#; reverse, 5#-AGGTGGCATCCCAGTTGTGG-3#. Western blotting of NEMO or actin was performed with 4% to 12% bis-Tris NuPage gradient gels, NuPage buffer systems, and polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked for nonspecific protein binding by the use of 1% BSA in phosphate buffered saline (PBS) with 0.1% Tween-20 for 1 h at room temperature, followed by overnight incubation with anti-NEMO or anti-actin antibodies.

The eight exons of *EDA-1* and *EDA-2* were amplified through PCR using the following primer sets, as previously described [20]: forward, 5#-GTCGGCCGGGACCTCCTC-3#; reverse 5#-GCCGCCGCCCCTACTAGG-3#; forward, 5#-ATGTTGGCTATGACTGAGTGG-3#; reverse, 5#-CCCTACCAAGAAGGTAGTTC-3#; forward, 5#-GATCCCTCCTAGTGACTATC-3#; reverse, 5#-CAGACAGACAATGCTGAAAGA-3#; forward, 5#-AAAAAAGTAACACTGAATCCTATT-3#; reverse 5#-CTCTCAGGATCACCCACTC-3#; forward, 5#-GGAAGTCAAAAGATTATGCCC-3#; reverse, 5#-CTACCCAGGAAGAGAGCAAT-3#; forward, 5#-CTGAGCAAGCAGCCATTACT-3#; reverse, GGGGAGAAGCTCCTCTTTG-3#; forward, 5#-ACTGAGTGACTGCCTTCTCT-3#; reverse, 5#-GCACCGGATCTGCATTCTGG-3#; forward, 5#-TGTCAATTCACCACAGGGAG-3#; reverse, 5#-CACAGCAGCACTTAGAGG-3#.

### 2.3. Immunological assays

PBMC was isolated from patients by density gradient centrifugation over Ficoll-Hypaque (Biochrom, Berlin, Germany). Cells were stained with the appropriate antibody (CD45-APC, CD3-PerCP, CD19-PerCP, CD56-PE-Cy7, CD8-PE-Cy7, CD4-FITC, CD27-APC, CD24-FITC, IgD-PE, IgM-PE, CD45RO-FITC [BD Biosciences, San Jose, California], CD45RA-PE, CD38-PE, CD31-PE [Miltenyi Biotec, Bologna, Italy]) at 4 °C for 30 min, washed and finally analyzed using a FACSCanto II flowcytometer (BD Biosciences).

The relative proportion of the following lymphocyte subpopulation was studied: T cells (CD3 +), helper T cells (CD3 + CD4 +), cytotoxic T cells (CD3 + CD8), B cells (CD3 − CD19 +), Natural Killer cells (CD3 − CD56 +), naïve helper T cells (CD3 + CD4 + CD45RA +), memory helper T cells (CD3 + CD4 + CD45RO +), naïve cytotoxic T cells (CD3 + CD8 + CD45RA +), memory cytotoxic T cells (CD3 + CD8 + CD45RO +), transitional B cells (CD3 − CD19 + CD24 + CD38hiCD27 −), mature B cells (CD3 − CD19 + CD24 − CD38dim/loCD27 −), IgM memory B cells (CD3 − CD19 + CD24 + IgM + CD27 +), and switched memory B cells (CD3 − CD19 + CD24 + IgM − CD27 +).

B cells were labeled with 5-chloromethylfluorescein diacetate at the final concentration of 0.1 mg/mL (Molecular Probes, Eugene, Oregon) and cultured at 1–2 × $10^5$ cells per well in 96 round-bottom plates (BD Biosciences) in complete RPMI-1640 (Invivogen, San Diego, California) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, Utah). Human CpG oligodeoxynucleotides (Hycult Biotechnology, Plymouth Meeting, Pennsylvania) was used at the optimal concentration of 2.5 mg/mL. Cell proliferation was measured on day 7 by a FACSCalibur Flow Cytometer (BD Biosciences). To evaluate B-cell function a lymphocyte gate based on forward and side scatter characteristics was used. The B cell gate was based on CD19 expression. The CMFDA fluorescence of CD19 + B cells was then evaluated. Secreted Ig was assessed at day 7 by ELISA. Briefly, 96-well plates (Corning Incorporated, New York, USA) were coated overnight with purified goat antihuman IgA, IgG or IgM (Jackson ImmunoResearch, West Grove, Pennsylvania). After washing with PBS/0.05% Tween and blocking with PBS/gelatin 1%, plates were incubated for 1 h with the supernatants of the cultured cells. After washing, plates were incubated for 1 h with peroxidase-conjugated fragment goat anti-human IgA, IgG or IgM antibodies (Jackson ImmunoResearch). The assay was developed with o-phenylenediamine tablets (Sigma, St Louis, Missouri) as a chromogenic substrate. Immunoglobulin concentration in the supernatants was measured by interpolation with the standard curve. As standard, human IgA, IgG and IgM (Jackson ImmunoResearch) were used. The standard curve was generated measuring seven successive 1:3 dilutions of each standard. Also for each supernatant seven successive 1:3 dilutions were tested.

The proliferation of PBMC was determined through the incorporation of tritiated thymidine during 72 h of culture after stimulation with 8 μg/mL PHA or CD3 cross-linking with anti-CD3 (1 or 0.1 ng/mL) monoclonal antibody precoated plates.

## 3. Results and discussion

### 3.1. Diagnosis of HED-ID or XLHED

Three male patients, of 5.0, 4.3 and 7.2 years of age were enrolled into the study. *IKBKG/NEMO* genetic analysis revealed a c.509T>C mutation in patient 1 and a c.1167dupT mutation in patient 2 (see Supplemental materials). While the c.1167dupT was previously found in a patient with HED-ID and osteopetrosis [21], the c.509T>C has been never reported before [5]. Patient 3 carried ac.1133C>T 140 mutation in EDA-1 gene. Array-Comparative Genomic Hybridization (Array-CGH) was performed in patient 2 and showed a partial trisomy of chromosome 22q11.1.

As shown in Supplemental Table I, all the patients had phenotypic hallmarks of HED at the diagnosis and reduced sweating after pilocarpine stimulation. Patients 2 and 3 suffered from recurrent impetiginized eczema requiring only topical therapy. Patient 2, also carrying a 22q11.1 trisomy, had further atypical dysmorphic features, as downward-sloping palpebral fissures, microstomia, retrognathism and elf ear and

showed a neurological involvement, characterized by reduced visual evoked potentials, mental retardation and pericerebral liquoral space dilatation on magnetic resonance imaging (Supplemental Table I). Although no conclusive explanation is available, developmental delay has been already associated with 22q11.1-q11.2 alteration [22]. Neurological manifestations may also be found in 30% of HED patients, representing one of the major causes of mortality of this disorder [23].

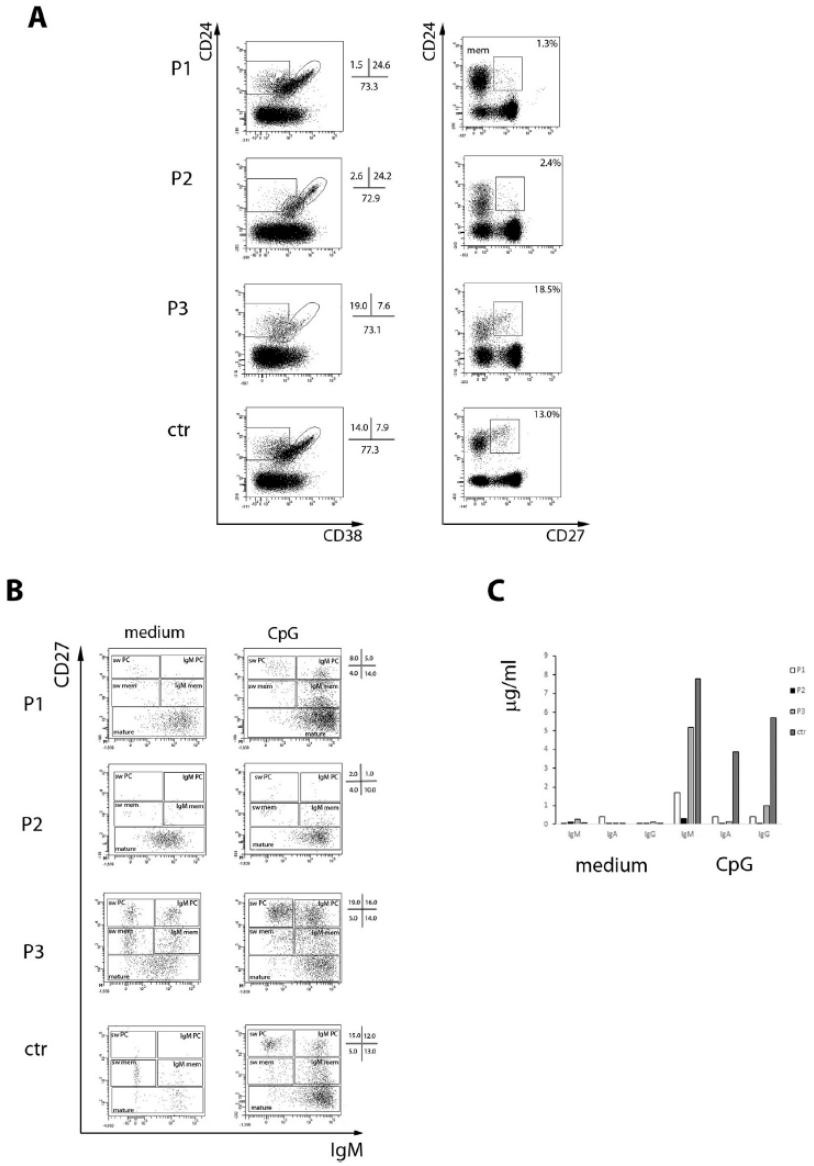


**Fig. 1.** B-cell phenotype and function of HED-ID and XLHED patients. (a)The left panel shows the identification of memory (CD3−CD19+CD24+CD27+), transitional (CD3−CD19+CD24+CD38hiCD27−), and mature B cells (CD3−CD19+CD24−CD38dim/loCD27−) in CD3−CD19+ gated cells using the CD24 and CD38 markers. In the right panel the staining for CD24 and CD27 in CD3−CD19+ gated cells is shown. The frequency of CD27+ memory B cells is indicated. (b)PBMCs cultured with medium or CpG were stained with antibodies to CD27 and IgM at day 7. Mature B cells are identified as IgM+, CD27− in CD3−CD19+ gated cells. IgM memory (IgM mem) B cells express IgM and CD27, whereas switched memory (Sw mem) lack IgM. Plasma cells are bright for CD27 and have (IgM PC) or lack (sw PC) IgM. (c)In the supernatants, IgM, IgG and IgA were measured by ELISA. Each column indicates the concentration measured in µg/mL.

### 3.2. Infections

In spite of the similarity of the cutaneous involvement, difference in the susceptibility to infections and in the immunological pattern, between HED-ID and XLHED patients, was documented. Patients 1 and 2, carrying *IKBKG/NEMO* alterations, suffered from severe infections including sepsis and severe gastroenteritis, requiring hospitalization and total parenteral nutrition. Patient 1 also suffered from *S. pneumoniae* meningitis and recurrent urinary tract infections. Recurrent upper and lower airway infections were reported in patients 1 and 3 (Supplemental Table II). Predisposition to bronchial infections has already been described in patients with XLHED. In these patients alterations of the airways seromucous glands have been identified and suspected to predispose to bronchial disease [24,25].

All patients suffered from chronic mucocutaneous candidiasis responsive to oral treatment. Fungal infections have been described in the 10% of the patients affected with HED-ID [11]. The pathogenesis of susceptibility to fungal infections in HED-ID has not been completely clarified. It should be noted that CARD-9 (caspase recruitment domain-containing protein 9), which plays a key role in immune response against fungal infections, activates NF-kB pathway (see Supplemental Fig. 1). This activation depends on the presence of NEMO for the recruitment of IKK complex, and NF-kB nuclear translocation and activity. This evidence supports a role for NEMO in the activation of innate anti-fungal immunity [26]. Interestingly, low IL-17 T cell counts have been shown in a patient with HED due to IKBA mutation, involved in Nf-kB signaling [27]. Notably, TLR is also implicated in the differentiation of IL-17 T cells, which play a very important role in the response to fungal infections [28]. As for XLHED patients, little is known about the predisposition to fungal infections. Even though according to some authors, the decreased salivary secretion could increase the risk of oral fungal infections [29], it is very difficult that such an explanation is per se sufficient to account also for the predisposition to esophageal infections, observed in our patient. In patients with XLHED, an immunodeficiency has never been described, even though the evidence of recurrent and often severe bacterial and fungal infections (pneumonitis and esophageal candidiasis) could suggest the presence of immunological alterations, which deserve further investigations on a larger cohort of patients.

### 3.3. Immunological findings

Leukocyte counts were persistently elevated in patient 1 with a median white blood cell (WBC) count at the initial diagnosis of 17,410 cells/μL. In patients 2 and 3 leukocyte counts were normal. All patients had normal CD3+, CD4+, CD8+, naïve and memory CD4 and CD8 T cells (see Supplemental Table III). The study of the B-cell compartment revealed a normal number of CD19+ cells in all the patients. As shown in Fig. 1A, patients 1 and 2 showed a marked reduction of CD19+CD24+CD27+ memory B cells, differently from patient 3 who had a normal memory B-cell subset but reduced transitional cells (Fig. 1a left panel). In patients 1 and 2, memory B cells mostly included IgM memory cells (95% and 80% of the memory B cells, respectively, data not shown). In patient 3, the relative proportion of switched and IgM memory was normal (52 and 48% of B cell memory, respectively, data not shown). As observed in other humoral immunodeficiencies, the defect of the B-cell compartment mainly involves terminal steps of B-cell differentiation [13,30].

In vitro B-cell differentiation and Ig production were studied stimulating PBMC with the TLR9 ligand, CpG. As previously reported [15], since the PBMC was analyzed after 7 days of culture, only a few memory B cells survived under unstimulated conditions (Fig. 1b, medium). As shown in Fig. 1B, B cells from the two patients carrying *IKBKG/NEMO* mutation proliferated in response to CpG, but they did not terminally differentiate into $CD27^{bright}$ plasma cells, differently from the control and patient 3. Consistently, IgG and IgA were not detectable in the supernatants and only small amounts of IgM were secreted in patients 1 and 2, differently from the control (Fig. 1c). Unexpectedly, memory Bcells from XLHED, which adequately differentiate into plasma cells, fail to produce normal amount of IgA or IgG upon TLR9 stimulation, as compared to the healthy control. Patients 1 and 2 had hypogammaglobulinemia, requiring IV-Ig therapy, differently from patient 3. Since 20 months of age, the patient 2 showed IgM levels >95th centile for age, which later normalized during the follow-up, while patient 1 since 2.9 years of age showed IgA levels >95th centile for age (data not shown). Isohemagglutinins were undetectable in patients 1 and 2. Only patient 3 had detectable hepatitis B-specific IgG (209.7 mIU/L). In patient 1, no specific antibody response to any of the 14 polysaccharide antigens was observed after immunization with pneumococcal polysaccharide vaccine, differently from patient 3, who had a normal response (Table 1). Of note, IgM memory B cells and natural antibodies have been shown to play an important role in the defense against encapsulated bacteria [14].

Lymphocyte proliferative response to PHA or stimulation through CD3 X-L was decreased only in patient 1, being 27.6 and 0.57% of the control, respectively.

In both HED-ID, no terminal differentiation of mature B cells into plasma cells and switched memory B cells was induced following TLR9 triggering, differently to what observed in XLHED. Moreover a marked reduction in the B-cell memory compartment was observed in both HED-ID patients.The difference between HED-ID and XLHED immune defect may rely on the distinct pathways in which NEMO and EDA-1 are involved. In fact, while both molecules are involved in the ectodysplasin pathway, thus explaining the similarity in the cutaneous involvement, NEMO is also involved in signaling pathways downstream to different receptors, including TLRs, IL1Rs, TNFRs, and B and T-cell receptors. The participation of NEMO to these pathways explains the complexity of immune defect in HED-ID.

In conclusion, in this study we report for the first time on an impaired B-cell differentiation in response to CpG signaling through TLR9, suggesting an alteration of the T-independent B-cell activation in HED-ID. This finding further expands the knowledge about the pathogenesis of humoral alterations in HED-ID patients and helps explain the high susceptibility to infections from encapsulated bacteria in such patients. Unexpectedly, also B cells from XLHED failed to produce normal amount of IgA or IgG, upon TLR9 stimulation, suggesting that an immunodeficiency may play a role in the predisposition to bronchial infections described in such patients. This finding needs to be confirmed in a study on a larger cohort. Moreover, in this study we found for the first time a new NEMO mutation (c.509T>C) in patient 1. This mutation was associated with the most severe immunodeficiency, suggesting a potential role for this alteration as a negative prognostic factor.

**Table 1**
Major immunologic findings of the patients.

| | Patient 1 | Patient 2 | Patient 3 |
|---|---|---|---|
| Immunoglobulins (mg/dL) | | | |
| IgG | 160 (231–947)[a] | 56 (222–846)[a] | 1410 (528–1959)[a] |
| IgA | 20 (8–74) | 6 (6–60) | 82 (37–257) |
| IgM | 10 (26–210) | 92 (28–39) | 77 (49–292) |
| IgE (KU/L) | <2 | NA | 1501 |
| Specific antibodies | | | |
| HBV antigen antibodies (mIU/mL) | Absent | Absent | 209.7 |
| Antibody response to pneumococcal polysaccharide (IgG/IgA/IgM)[b] | | | |
| Pre | 271/32/302 | NA | 88/75/94 |
| Post | 237/36/200 | NA | 280/286/302 |
| Isohemagglutinins | Absent | Absent | Anti-A 1:64[c] Anti-B 1:256 |

NA,not available.
[a] Normal reference value.
[b] ng/mL; a positive response is defined as a threefold increase of the antibody titer.
[c] Anti-A and anti-B isohemagglutinin titers.

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ARTICLE

# Unraveling the Link Between Ectodermal Disorders and Primary Immunodeficiencies

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Primary immunodeficiencies (PIDs) include a heterogeneous group of mostly monogenic diseases characterized by functional/developmental alterations of the immune system. Skin and skin annexa abnormalities may be a warning sign of immunodeficiency, since both epidermal and thymic epithelium have ectodermal origin. In this review, we will focus on the most common immune disorders associated with ectodermal alterations. Elevated IgE levels represent the immunological hallmark of hyper-IgE syndrome, characterized by severe eczema and susceptibility to infections. Ectodermal dysplasia (ED) is a group of rare disorders that affect tissues of ectodermal origin. Hypoidrotic ED (HED), the most common form, is inherited as autosomal dominant, autosomal recessive or X-linked trait (XLHED). HED and XLHED are caused by mutations in *NEMO* and *EDA-1* genes, respectively, and show similarities in the cutaneous involvement but differences in the susceptibility to infections and immunological phenotype. Alterations in the transcription factor *FOXN1* gene, expressed in the mature thymic and skin epithelia, are responsible for human and murine athymia and prevent the development of the T-cell compartment associated to ectodermal abnormalities such as alopecia and nail dystrophy. The association between developmental abnormalities of the skin and immunodeficiencies suggest a role of the skin as a primary lymphoid organ. Recently, it has been demonstrated that a co-culture of human skin-derived keratinocytes and fibroblasts, in the absence of thymic components, can support the survival of human haematopoietic stem cells and their differentiation into T-lineage committed cells.

**Keywords:** ectodermal dysplasia, *FOXN1*, hyper-IgE, primary immunodeficiencies, T-cell development

## INTRODUCTION

Primary immunodeficiencies (PIDs) include a heterogeneous group of diseases, mostly monogenic, which are characterized by functional/developmental alterations of the immune system. In the last decades, the field of PID has been deeply studied, eventually leading to an overall better knowledge and nosografic re-classification of the different forms so far identified. In particular, several novel forms have been described unraveling new clinical and genetic aspects. Nevertheless, it has been documented that an inappropriate or late diagnosis of PID by clinicians often occurs, thus indicating the strong need of an update on the novel clinical associations of different forms and alarm signals. An overview on this topic would favor an early diagnosis.

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Recent evidence highlights that the skin participates in a host defenses either acting as a primary boundary for germs, as the principal site of environment–host interactions, or directly in the developmental process of the immune system. As a matter of fact, skin and skin annexa abnormalities, such as skin dryness, brittleness of hair, nail abnormalities and abnormal dentition, can be not infrequently associated with distinct forms of immunodeficiency.

In this review, we will focus on the link between skin developmental alterations and PIDs that could help in the early detection of some immunologic disorders.

## ECTODERMAL DYSPLASIA AND IMMUNE DEFECTS

Ectodermal dysplasia (ED) is a group of rare inherited disorders that affect two or more tissues of ectodermal origin. Ectodermal dysplasia has an incidence of seven cases per 10,000 live births and to date nearly 200 different forms of ectodermal dysplasia have been described. The main abnormalities involve the skin, which may be dry, thin and hypopigmented, and prone to rashes, eczema or infections. Furthermore, sweat glands may function abnormally, or may not develop at all, and hair is usually hypopigmented, thin and sparse.

The abnormal sweat production may impair body temperature control, thus leading to overheating, especially in hot environments. Airways seromucous glands may also be affected predisposing to respiratory infections because of the absence of the normal protective secretions of the mouth and nose. Defect in meibomian/tarsal glands may lead to dryness of the eye, cataracts, and vision defects. Teeth may be congenitally absent, peg-shaped or pointed. The enamel may also be defective. Typical cranial-facial features include frontal bossing, longer or more pronounced chins and broader noses. Abnormalities in the ear development may cause hearing problems.

Hypoidrotic ED (HED) is the most common form with an incidence of 1:10,000 [1]. This form is inherited as an autosomal dominant (AD), autosomal recessive (AR) or X-linked trait (XLHED). HED derives from mutations in the ectodysplasin-A (EDA) signaling pathway, which leads to the expression of genes implicated in the development of the skin and skin appendage. Mutations in the *EDA* gene on X-chromosome cause approximately 80% of cases of HED (OMIM 305100, XLHED, ectodermal dysplasia, type 1, ED1). A smaller subset of cases is caused by mutations in the EDA receptor (EDAR), the adapter protein (EDARADD), or WNT10A [2, 3], being inherited in an autosomal recessive (ectodermal dysplasia anhidrotic; EDA; OMIM 224900) or autosomal dominant manner (ectodermal dysplasia type 3; ED3; OMIM 129490). EDA regulates organogenesis at multiple levels, from the initiation to the terminal differentiation [4, 5]. The activation of EDA pathway is implicated in the appearance of focal thickenings of the epithelium known as placodes, implicated in the very early stage of skin appendage development. *EDA* gene is a member of the TNF superfamily. EDA ligand binds to the trimeric EDAR receptor, which, in turn upon binding, recruits the EDARADD adaptor via death-domain–death-domain interactions. This cascade, from EDA and EDAR to EDARADD, leads to the activation of the NF-kB pathway. In unstimulated cells, NF-kB is sequestered in the cytoplasm by the inhibitor of the kBproteins (IkB). NF-kB essential modulator (NEMO), also called IkB Kinase (IKK) gamma protein, acts as a regulatory subunit of the IKK complex, comprising two kinase subunits (IKK1/a and IKK2/b), required for the activation of canonical NF-kB pathway. Upon stimuli, IkB is phosphorylated by the IKK, resulting in IkB degradation, NF-kB translocation into the nucleus, and eventually regulation of the target gene expression [6].

Hypomorphic mutations in the NEMO encoded by the *IKBKG/NEMO* gene on the X-chromosome result in HED with immunodeficiency (HED-ID, OMIM 300291) [7–9].

In spite of the similarity of the cutaneous involvement, difference in the susceptibility to infections and in the immunological pattern between HED-ID and XLHED patients is well documented. Due to the pleiotropic role of NEMO, mutations in *IKBKG/NEMO* gene lead to a heterogeneous and severe immunodeficiency. In patients with XLHED, immunologic alterations have never been reported. However, in these patients recurrent bronchial or eye infections have been described and interpreted as a result of reduced bronchial or meibomian/tarsal gland function. The difference between HED-ID and XLHED immune defect may rely on the distinct pathways in which NEMO and EDA-1 are involved. In fact, while both molecules are involved in the ectodysplasin pathway, thus explaining the similarity in the cutaneous involvement, NEMO is also involved in signaling pathways downstream to different receptors, including toll-like (TLRs), interleukin-1 (IL-1Rs), tumor necrosis factor (TNFRs), and B- and T-cell receptors (TCR and BCR). The participation of NEMO in these pathways explains the wider immune defect in HED-ID and its complexity. HED-ID is characterized by unusually severe, recurrent, and sometimes life-threatening bacterial infections of the lower respiratory tract, skin, soft tissues, bones and gastrointestinal apparatus as well as meningitis and septicemia in early childhood. These patients display high susceptibility to infections by Gram-positive bacteria (*S. pneumoniae* and *S. aureus*), followed by Gram-negative bacteria (*Pseudomonas* spp. and *Haemophilus influenzae*) and mycobacteria, as well.

Laboratory features include hypogammaglobulinaemia with low serum IgG (or IgG2) levels, and variable levels of other immunoglobulin isotypes (IgA, IgM and IgE). Elevated serum IgM levels have been described in a number of HED-ID patients with the hyper-IgM6 phenotype [10, 11]. In some patients, a defective ability of B cells to switch in response to CD40 ligand (CD40L) has been described, which may help explain the hyper-IgM phenotype. Defective antibody response to polysaccharide and proteic antigens is the most consistent laboratory feature. Recently, impaired NK activity has also been reported in some [12] but not all [13] patients with EDA-ID. NEMO also acts downstream to TLRs [13–15]. As a consequence, NEMO patients exhibit poor inflammatory response, also due to impaired cellular responses to pro-inflammatory cytokines (IL-1$\beta$, IL-18 and TNF-$\alpha$) [15]. Impaired IL-1$\beta$- and IL-18-dependent induction of IFN-$\gamma$, impaired cellular responses to IFN-$\gamma$-inducible TNF-$\alpha$, and impaired signaling through TLRS may explain the occurrence of severe mycobacterial disease in these patients.

Different *NEMO* mutations have also been associated with distinct disorders. While loss-of-function mutations cause incontinentia pigmenti (IP), hypomorphic mutations cause two allelic conditions, namely HED-ID and a clinically more severe syndrome, in which osteopetrosis and/or lymphoedema associate with HED-ID (OL-HED-ID; MIM 300301). Mutations in the coding region are associated with the HED-ID phenotype (MIM 300291), while stop codon mutations cause a OL-HED-ID [11, 16–21]. IP (OMIM#308300), which specifically affects females, being lethal in males [22], is caused by a complex rearrangement of *NEMO* gene that results in the deletion of exons 4–10, coding for a shortened protein unable to elicit an NF-$\kappa$B response. This recurrent rearrangement accounts for 85% of IP patients [16, 17]. Affected females present with Blashko linear skin lesions [23] variably associated with developmental anomalies of teeth, eyes, hair and the central nervous system.

Mutations in other genes involved in NF-$\kappa$B pathway are responsible for different forms of HED-ID. Gain-of-function mutations of I$\kappa$B$\alpha$ are able to enhance the inhibitory capacity of I$\kappa$B$\alpha$ through the prevention of its phosphorylation and degradation, and result in impaired NF-$\kappa$B activation leading to HED-ID. The developmental, immunologic and infectious phenotypes associated with hypomorphic NEMO and hypermorphic IKBA mutations largely overlap and include EDA, impaired cellular

**TABLE 1.** Clinical and immunological features of hyper-IgE syndrome (HIES).

| Immunodeficiency | Gene | Immunological phenotype | Clinical features | OMIM |
|---|---|---|---|---|
| HIES-AD | *STAT3* | Reduced Th17 lymphocytes; reduced specific antibody response; reduced switched and no switched B memory lymphocytes | Facial anomalies, eczema, osteoporosis and pathological fractures, teeth anomalies, joint laxity, Stafilococcus aureus infections (pulmonary and cutaneous abscesses, pneumatocele), candidiasis | #147060 |
| HIES-AR | *TYK2* | Altered cytokine signaling | Increased susceptibility to fungi, viruses and intracellular bacteria (mycobacterium, salmonella spp.) | #611521 |
| | *DOCK8* | Reduced T, B and NK cells, hyper-IgE, reduced IgM levels | Severe atopy, hypereosinophilia, recurrent infections, severe viral and bacterial cutaneous infections, predisposition to cancer | #243700 |

responses to ligands of TIR (TLR-ligands, IL-1$\beta$ and IL-18), and TNFR (TNF-$\alpha$, LT$\alpha$1/$\beta$2 and CD154) super family members leading to severe bacterial diseases.

Recently, mutations in *NF-kB2* gene have been described as responsible for the early onset of common variable immunodeficiency, inherited as an autosomal dominant trait. In the cases so far identified, ectodermal abnormalities, including nail distrophy and alopecia together with endocrine alterations, have been reported.

## PRIMARY IMMUNODEFICIENCIES WITH HYPER-IgE

Elevated IgE levels represent the immunological hallmark of a growing group of PID termed as hyper-IgE syndrome (HIES). The clinical phenotype of these syndromes, along with very high IgE levels (>2000 IU/L), comprises severe eczema and susceptibility to a spectrum of infections, especially staphylococcal and fungal infections, involving the skin and lungs. These disorders can be inherited in an autosomal dominant or autosomal recessive manner (Table 1). Sometimes, sporadic cases have been described. It isn't always easy to differentiate the syndromes from the severe forms of atopic dermatitis, in which high levels of serum IgE, and sometimes viral or bacterial infections, could also occur, since the complete clinical phenotype of HIES often becomes evident only over years. This may cause delay in diagnosis, especially in those patients who have milder forms of the disease.

In 2007, Holland et al. [24] found that hypomorphic mutations of signal transducer and activator of transcription 3 (*STAT3*) gene are responsible for the autosomal dominant form of HIES, characterized by the classic clinical triad represented by recurrent cutaneous "cold" abscesses, recurrent pulmonary infections and increased concentration of serum IgE. Such triad is present in 75% of autosomal dominant cases and in 85% of children with a disease onset before 8 years of age. In many cases, eczema, often with a neonatal onset, is the first sign of the disease.

In the patients with a *STAT3* defect, in addition to Staphylococcus aureus, often methicillin-resistant, infections with other pathogens, such as Haemophilus influenzae and Streptococcus pneumonia, may also be found. Moreover, the recurrent sinopulmonary infections, through the formation of bronchiectasis and some-

times pneumatoceles, represent predisposing factor to colonization by opportunistic agents such as Pseudomonas aeruginosa and Aspergillus fumigatus, with the risk of developing invasive aspergillosis and systemic fatal infections. Another frequent infection is chronic mucocutaneous candidiasis and, to a lesser extent, Pneumocystis jirovecii lung infection. In addition, other fungal pathogens, including Histoplasma, Coccidioides and Cryptococcus, have been reported to cause gastrointestinal infections as well as meningitis in patients with HIES [25]. This increased susceptibility to infections is due to the impairment of Th17-cell function through the alteration of signaling mediated by various cytokines, and in particular IL-6 and IL-22 [26]. Beyond the immunological and infectious features, patients also exhibit different non-immunological features, including craniofacial, neurological, dental, vascular and musculoskeletal anomalies.

In 2004, Renner et al. [30] have described the case of a novel form of HIES, sharing some of the clinical features of the AD-HIES, but with an AR inheritance and a different profile of susceptibility to infections. In addition, these patients have a neurological involvement and a high predisposition to autoimmunity and proliferative disorders. The genetic defect was first identified in 2006, with the recognition of mutations in the *TYK2* gene [27]. In particular, the patient suffering from this variant showed alterations of the signaling pathway mediated by IFN$\alpha$, IL-6, IL-10, IL-12 and IL23, resulting in an impairment of both innate and adaptive immunity. The TYK2 deficiency remains, however, a very rare form, whose clinical features are still controversial, as demonstrated from the description of the second case, with very different clinical presentation, characterized by disseminated BCG infection, recurrent zoster and neurobrucellosis in the absence of high levels of IgE [28].

On the other hand, many cases of AR-HIES have been ascribed to alterations in the *DOCK8* gene, which encodes a protein involved in the regulation of cytoskeleton [29]. Patients with *DOCK8* deficiency show a more severe phenotype than AD-HIES patients, characterized by severe viral infections primarily involving the skin (HPV, VZV, MCV), recurrent bacterial infections, severe atopy and high risk of early onset malignancies to the extent of 10–36% of patients. Notably, *DOCK8* deficiency may be associated with IgE levels within the normal range or only moderately elevated as compared with AD-HIES form. In addition, AR-HIES patients do not display somatic features, such as dental abnormalities, craniofacial or skeletal abnormalities, compared with the STAT3-dependent AD-HIES [30]. Eventually, neurological manifestations, such as facial paralysis, hemiplegia, cerebral aneurisms, and CNS vasculitis have been observed [30]. Within the malignancies, HPV-associated carcinomas, EBV-associated Burkitt lymphoma and diffuse large B cell lymphoma clearly predominate, not infrequently with an onset during the childhood [31].

A recent study described the case of a patient with Olmsted Syndrome due to transient receptor potential cation channel, subfamily V, member 3 (*TRPV3*) gene mutations, characterized by hyperkeratotic cutaneous lesions and palmoplantar keratoderma associated with a peculiar immunological and infectious phenotype characterized by high IgE levels, recurrent hypereosinophilia, increased IgA levels, reduced IgG3 subclasses and frequent skin infections sustained by bacteria and fungi, particularly by Candida albicans. The clinical phenotype is highly suggestive of a primary role of *TRPV3* gene, which is expressed in keratinocytes and langerhans cells of the skin in the immune response [32].

As a matter of fact, elevated serum IgE levels, although at a lower extent than HIES, are often found in many other PIDs, including Omenn Syndrome, due to hypomorphic mutations of *RAG1*, *RAG2*, *ARTEMIS*, *ADA* and *RMRP* genes; Wiskott–Aldrich Syndrome due to mutations in the *WAS* gene; atypical DiGeorge Syndrome with deletion of chromosome 22q11.2; IPEX Syndrome (immuno-disregulation,

polyendocrinopathy, enteropathy, X-linked) caused by mutation of the *FOXP3* gene and finally, Comel–Netherton Syndrome due to defect of *SPINK5* (Table 2) [33]. Each of these disorders shows a peculiar clinical phenotype that strongly distinguishes them from the classical forms of HIES. Such a strong association between the number of PIDs, whichever is the form, and the elevated IgE levels, would argue in favor of a still unappreciated biologic role for IgE in these patients and, in general, in the immune system physiology.

## NUDE/SCID PHENOTYPE

### Ectodermal Disorders and *FOXN1* Transcription Factor

As previously mentioned, ectodermal dysplasias include disorders sharing abnormalities of the skin, its appendages, such as hair, nails, teeth, sweat glands and sebaceous glands, and of other organs, which develop from ectoderm, such as the nervous system, the lens of the eye, and the mammary glands. These disorders may appear separately or together with other clinical manifestations involving mesoderm and endoderm [7]. Due to the huge number of different ectodermal dysplasias, there is a remarkable overlapping of clinical phenotypes.

A few overlapping signs with ectodermal dysplasia, associated with immunological abnormalities, are found in immunodeficiencies, such as those caused by the alterations of the *NEMO* gene [34], and of the RNA component of mitochondrial RNA processing endoribonuclease gene (*RMRP*), responsible for cartilage hair hypoplasia syndrome [35], or the *FOXN1* gene, the latter being responsible for the human or murine athymia, associated with the skin and hair defects, and, putatively, neural tube abnormal development [36, 37]. *FOXN1* gene is a "winged helix" transcription factor belonging to the forkhead-box gene family, which comprises genes implicated in a variety of cellular processes, such as development, metabolism, cancer and aging [38]. These transcription factors are developmentally regulated and direct tissue-specific transcription and cell fate decisions. In the pre-natal life, *FOXN1* is expressed in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney, and urinary tract, while in the post-natal life *FOXN1* is expressed only in the epithelial cells of the skin and thymus. In the epidermis, *FOXN1* is expressed within differentiated epidermal and follicular cells. Differently, in the thymus, *FOXN1* acts early in the organ development, promoting TEC progenitor proliferation and directing specification of thymic epithelial precursor cells to cortical and medullary lineages [39–42]. The tissue specificity expression of *FOXN1* is probably due to the presence in its sequence of two exons, exons 1a and 1b, that undergo to alternative splicing to either of the two splice acceptor sites of the exon 2. The alternative usage of exon 1a or 1b is due to the presence of distinct promoters: promoter 1a, which is active in both the thymus and the skin, and promoter 1b, which is active only in the skin [43].

### The Nude/SCID Syndrome and Its Associated Skin Abnormalities

The human Nude/SCID syndrome is characterized by the absence of a functional thymus, which results in a severe T-cell immunodeficiency [36]. This phenotype is the first example of SCID due to mutations of gene not expressed in hematopoietic cells [44].

Studies performed on human Nude/SCID fetus have added novel information on T-cell development in humans and, in particular, on the crucial role of *FOXN1* in early prenatal stages of T-cell ontogeny in humans. *FOXN1* gene mutations prevent the development of the T-cell compartment, affecting the $CD4^+$ cells more than the $CD8^+$ ones, as early as at 16 weeks of gestation [45]. Of note, in the absence of *FOXN1*, the thymic functionality is almost absent, as demonstrated by the absence of $CD4^+CD45RA^+$ naive cells [45]. However, very few $CD3^+CD8^+CD45RA^+$ naive cells

**TABLE 2.** Primary immunodeficiencies with elevated IgE levels.

| Immuno deficiency | Inheritance | Gene | Immunological phenotype | Clinical features | OMIM |
|---|---|---|---|---|---|
| Omenn Syndrome | AR | Hypomorphic mutations of *RAG1/2*, *ARTEMIS*, *ADA* and *RMRP*, *IL7/Ra*, *DNA ligase IV*, $\gamma c$, other unknown genes | Elevated IgE, reduced serum Ig levels, normal number of T lymphocytes with low heterogeneity, normal or reduced B lymphocytes | Erytroderma, eosinophilia, lymphoadenopathy, hepatosplenomegaly | #603554 |
| Wiskott–Aldrich Syndrome | XL | *WAS* | Increased IgA and IgE, altered lymphocytic proliferative response, no or/low antibody response to polysaccaride antigens | Microtrombocytopenia, eczema, autoimmune disorders; viral and bacterial infections | #301000 |
| Wiskott–Aldrich type 2 | AR | *WIPF1* | Reduced B and T CD8 lymphocytes, low NK activity | Eczema, trombocytopenia, recurrent infections | #614493 |
| Comel–Netherton Syndrome | AR | *SPINK5* | High IgE, reduced IgA levels, reduced switched and no-switched B lymphocytes | Ichtyosis, bamboo hair, atopy, increased susceptibility to viral and bacterial infections, growth retardation | #256500 |
| IPEX | XL | *FOXP3* | Altered number and/or function of regulatory CD4+ CD25+ FOXP3+ T-cells, normal or elevated IgA and IgE levels | Autoimmune enteropathy, early-onset diabetes mellitus, eczema, autoimmune disorders | #304790 |
| Olmsted Syndrome | AR | *TRPV3* | High IgE and IgA levels, reduced IgG3, hypereosinophilia | Palmo-plantar keratoderma, alopecia, onichodistrophy, recurrent fungal and bacterial cutaneous infections, squamous cell carcinoma | #614594 |

can be detected in the peripheral blood. Most of the T-cells bear TCR$\gamma\delta$ instead of TCR$\alpha\beta$ [45] and, although altered, the TCR gene rearrangement occurs in the absence of the thymus, suggesting an extrathymic site of differentiation for TCR chains, which is *FOXN1*-independent. Indeed, recent evidence suggests that, during embryogenesis, in the absence of *FOXN1*, a partial T-cell development can occur at extrathymic sites [46].

The Nude/SCID syndrome is more severe than the DiGeorge Syndrome, an immunodeficiency due to a complete or partial absence of the thymus, not associated with hairlessness or gross abnormalities in skin annexa. Peculiar features of the Nude/SCID syndrome are ectodermal abnormalities, such as alopecia and nail dystrophy [47]. The first identified mutation of *FOXN1* gene responsible in homozigosity for the disease is the C-to-T shift at 792 nucleotide position in the exon 4 (formerly exon 5) of the cDNA sequence. This mutation results in a non-sense mutation (R255X) and the complete absence of protein [48]. The second one, described in a French patient, is the C987T transition in exon 6, resulting in a mis-sense mutation (R320W) of the DNA binding domain [49]. A third novel mutation in *FOXN1*, resulting in TlowB+NK+SCID with alopecia [50] has been described recently.

The first two patients, identified by Pignata et al., in 1996, were two sisters with T-cell immunodeficiency and alopecia of the scalp, eyebrows, eyelashes and nail dystrophy [36]. Alopecia and nail dystrophy are also the characteristics of dyskeratosis congenita (DC) [51, 52], whose diagnostic criteria include a reticular pattern of hyper- and hypopigmentation of the skin, nail dystrophy, and mucosal leucoplakia [53]. However, in the two patients described, two diagnostic criteria of DC (abnormal pigmentation of the skin and mucosal leucoplakia) were lacking. Moreover, also the immunologic abnormalities were different from those associated with DC [54, 55].

The causal relationship between alopecia, nail dystrophy, and immunodeficiency does exist in Nude/SCID patients, and is also found in athymic mice that completely lack body hair (Table 3). Mice homozygous for the *FOXN1* mutation have retarded growth, decreased fertility, die of infections and are hairless (from which the term "nude" derived). The hairless feature is due to an abnormal epidermal developmental process, since in the skin of the nude mouse there are a normal number of hair follicles but not capable to enter the skin surface [56, 57]. In addition, the epidermis of the nude mouse fails to differentiate the spinous, granular and basal layers and shows a reduced number of tonofilaments. As in humans, also in mice the thymus is absent at birth [58], thus leading to a profound T-cell deficiency, which also affects humoral immunity. Of note, in a few strains of nude mice, alterations of digits and nails have been reported.

In 1999, a screening search for this *FOXN1* mutation in order to provide genetic counseling and prenatal diagnosis support to the community where the first patients were identified, led to the identification of healthy subjects carrying the heterozygous *FOXN1* mutation. These subjects were further examined for ectodermal alterations and showed nail abnormalities, such as the koilonychia (spoon nail), characterized by a concave surface and raised edges of the nail plate and the canaliform dystrophy associated to a transverse groove of the nail plate (Beau line), and the leukonychia (half-moon), characterized by a typical arciform pattern involving the proximal part of the nail plate [47]. This is not surprising, since *FOXN1* is selectively expressed in the nail matrix where the nail plate originates, and where it is involved in the maturation process of nails. In keeping with the expression of *FOXN1* in the murine epithelial cells of the developing choroids plexus, a structure filling the lateral, third, and fourth ventricles, additional studies revealed, in human Nude/SCID aborted fetus, the presence of severe neural tube defects, including anencephaly and spina bifida. However, since the anomalies of brain structures have been reported only inconstantly, this

**TABLE 3.** Main similarities shared between human Nude/SCID and murine "nude" phenotype.

| | Human Nude/SCID | Nude mouse |
|---|---|---|
| *Clinical features* | | |
| Thymus absence | + | + |
| Retarded growth | + | + |
| Omen-like syndrome | + | - |
| Severe infections | + (interstitial pneumopathy) | + |
| Neural tube defects | + (anencephaly and spina bifida) | - |
| Severe infertility | Unknown | + |
| Small ovaries with low eggs count (female) | Unknown | + |
| Motile sperm absence (male) | Unknown | + |
| Altered serum levels of estradiol, progesterone and thyroxine | Unknown | + |
| *Immunological features* | | |
| Presence of normal T-cell precursors | + | + |
| Lymphopenia | + (T-cells) | + (T-cells) |
| Absence of specific thymus-derived cells | + | + |
| Absence of proliferative response to mitogens | + | + |
| Very few lymphocytes in the thymus-dependent areas of the spleen and the lymph node | + | + |
| Presence of antibody forming cell precursors | + | + |
| Low levels of serum immunoglobulins | + | + |
| Very low/absent production of specific antibodies | + | + |
| *Skin and skin annexa features* | | |
| Hairlessness | + (alopecia of the scalp, eyebrows and eyelashes) | + |
| Alterations of digits and nails | + (leukonychia, koilonychias, canaliform dystrophy) | + |
| Unbalance between proliferation and differentiation of keratinocytes in the hair follicle | + | + |
| Coiling of incomplete hair shafts in the dermis | + | + |

would suggest that *FOXN1* plays a role of a cofactor only in brain development during embryogenesis [37].

## SKIN ELEMENTS TO SUPPORT T-CELL ONTOGENY

Given the association between skin developmental alterations and immunodeficiencies, a possible explanation is that a remarkable number of similarities are shared between the epidermal and the thymic epithelium. Similarities between the human thymic epithelial cells (TECs), a key cell component of the thymic stroma, and human

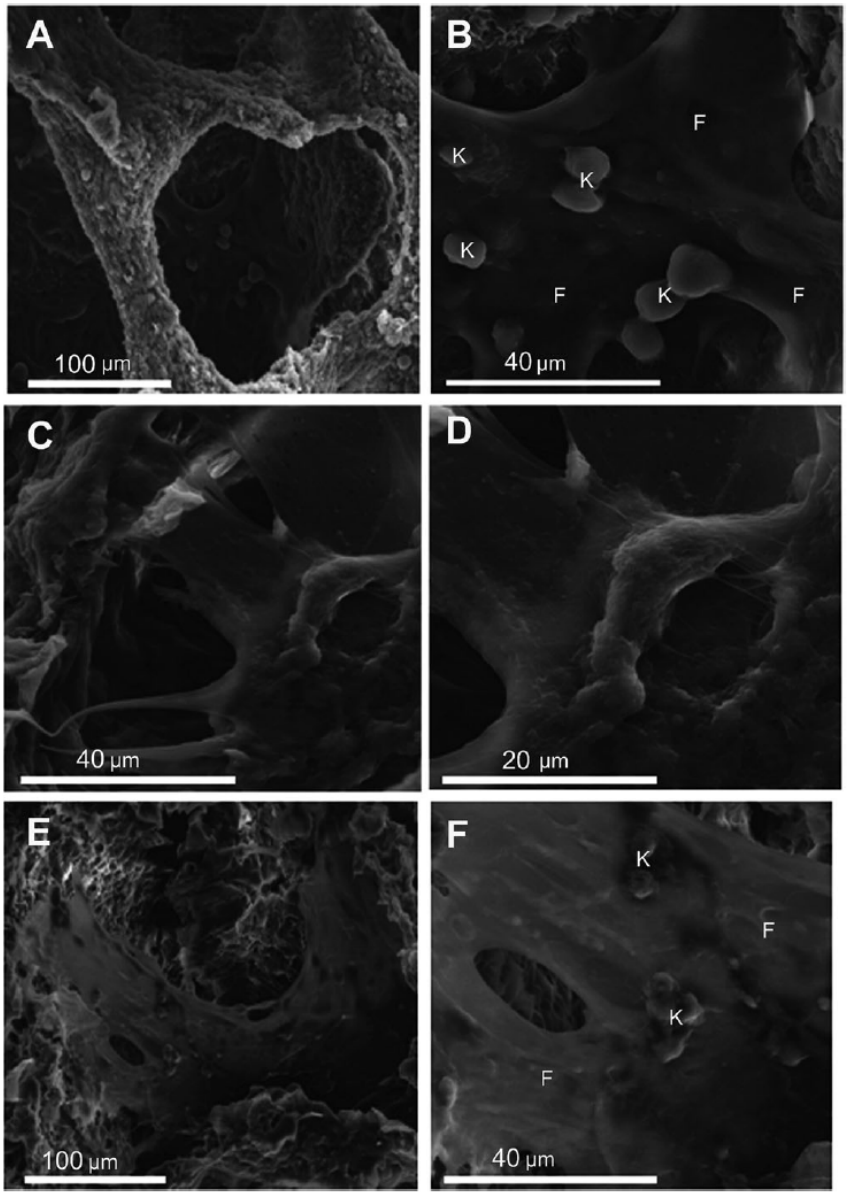


**FIGURE 1.** Representative scanning electron micrographs of keratinocytes and fibroblasts co-cultured on the PCL scaffold. Expanded fibroblasts and keratinocytes were seeded together onto artificial 3D PCL scaffolds, and after cell infiltration, the structure was studied by scanning electron microscopy (SEM). (A) Each cell type established physical contacts with the PCL scaffold and organized on the surface of its inner pores. (B) In particular, a strong interaction of keratinocytes with fibroblasts occurred. (C and D) Fibroblasts formed focal adhesions with the matrix, thanks to their thin filopodi (D). After 3 weeks of culture, interaction of each cell type with material (E) and with each other (F) was clearly visible.

keratinocytes were identified through comparison of gene and protein expression and *in vitro* analysis [59, 60]. The development of both epidermal and thymic epithelium requires the expression of p63, the p53 family transcription factor [61–63], which was earlier expressed in the development of both epithelial lineages [63–65]. Epidermal development is abrogated in mouse models with the loss of p63 function, resulting in few keratinocytes and lack of stratification, which causes rapid dehydration and early postnatal lethality of these mice [66, 67]. A similar epithelial phenotype occurs in p63−/− mice's thymus, which showed defects in the proliferative rate of TECs that leads to thymic atrophy.

Along with *FOXN1*, another transcription factor is shared between epidermal progenitor cells in the epidermis and the thymus, the T box gene *Tbx1*. The absence of *Tbx1* results in the loss of hair follicle stem cell renewal in the epidermis [68], and in the loss of thymic epithelial development [69–71]. Furthermore, thymic stroma and skin elements also share the Notch pathway, which plays an important role in the regulation of epidermal differentiation [72]. Within the thymus, it is necessary for T-cell linage commitment and the early stage of thymocyte maturation [73].

A major regulator of both hair follicle placode formation and thymic epithelial development is the fibroblast growth factor (FGF) signaling pathway [74, 75]. Several studies revealed the importance of FGFs mesenchymal expression to promote epithelial proliferation and invagination to generate mature thymic rudiments and epidermal hair placodes [76, 77].

Additional similarities between the two organs concern the cellular organization, in that medullary TECs are able to form Hassall's corpuscles, following a developmental program, analogous to skin epidermal basal cells, which form cornified cells [60]. Furthermore, proliferating TECs, derived from rats transplanted into the skin, formed epidermis and skin appendages such as the sebaceous gland and hair follicle [61], highlighting the responsiveness of TECs to the skin tissue environment.

Thus, although the functions of the skin and thymic epithelial components are quite distinct, both tissues have primary roles in establishing immunity [78]. TECs create an environment that promotes the expansion, maturation and specification of immature T cells. Epidermal keratinocytes are also essential for driving the activation of the innate and adaptive immune system through the production of cytokines, which direct the fate of discrete lymphocyte populations, known as the "epimmunome" [79].

Not by chance, recently it has been demonstrated that a co-culture of human skin-derived keratinocytes and fibroblasts, in the absence of thymic components, can support the survival of human hematopoietic stem cells and their differentiation into T-lineage committed cells [80], suggesting that skin keratinocytes can promote T-cell development, such as TECs, although at a low efficiency (Figure 1).

In addition, it has been shown that murine skin fibroblasts, enforced by *FOXN1* expression, are able to reprogram into induced TECs (iTECS), an *in vitro* generated cell type that exhibits phenotypic and functional properties of *in vivo* TECs. iTECS are able to promote full T-cell development *in vitro*, providing the basis for thymus transplantation therapies aimed at boosting adaptative immune system function in immunocompromised patients [81].

## CONCLUSIONS

Primary immunodeficiencies are severe early onset immunological disorders often fatal in the first years of life. Many forms show cutaneous features in association with immunological defects. In fact, the presence of skin and skin annexa abnormalities may be considered a warning sign in patients with a suspicion of a primary immunodeficiency. In this review, we focused on the most common forms of PIDs associated with

ectodermal disorders, highlighting the alarm signs that should lead the clinician to consider a deeper immunological assessment, investigating both molecular and functional aspects. Moreover, this approach would be very helpful in the early detection and treatment of such complex disorders.

### Declaration of Interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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# Novel Findings into AIRE Genetics and Functioning: Clinical Implications

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Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), formerly known as autoimmune polyendocrine syndrome type 1, is a paradigm of a monogenic autoimmune disease caused by mutations of a gene, named autoimmune regulator (*AIRE*). AIRE acts as a transcription regulator that promotes immunological central tolerance by inducing the ectopic thymic expression of many tissue-specific antigens. Although the syndrome is a monogenic disease, it is characterized by a wide variability of the clinical expression with no significant correlation between genotype and phenotype. Indeed, many aspects regarding the exact role of AIRE and APECED pathogenesis still remain unraveled. In the last decades, several studies in APECED and in its mouse experimental counterpart have revealed new insights on how immune system learns self-tolerance. Moreover, novel interesting findings have extended our understanding of AIRE's function and regulation thus improving our knowledge on the pathogenesis of APECED. In this review, we will summarize recent novelties on molecular mechanisms underlying the development of APECED and their clinical implications.

**Keywords: APECED, autoimmune disease, diagnosis, AIRE, mutations**

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## INTRODUCTION

Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), formerly known as autoimmune polyendocrine syndrome type 1 (APS-1), is a rare disease caused by mutations of the autoimmune regulator (AIRE) which acts as a transcription regulator that promotes immunological central tolerance (1).

Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy represents a paradigm of genetically determined systemic autoimmunity. However, the great variability that characterizes APECED, irrespectively of AIRE genotype, implies that additional factors modulate the clinical expression of the disease.

Recent advances on how AIRE affects immunological tolerance and is linked to organ-specific autoimmunity have improved our understanding on the pathogenesis and the wide variability of clinical expression of APECED.

In this review, we will summarize new insights into AIRE genetics and functioning and its implications on APECED phenotype.

## NEW INSIGHTS INTO AIRE FUNCTION

Autoimmune regulator is known to exert a crucial role in central tolerance and negative selection of autoreactive T cells (1). The induction of central tolerance is an intricate process that occurs

within the thymus where immature T lymphocytes are "committed" to become mature cells able to respond to a huge number of foreign antigens, but preventing autoimmune reactions. Medullary thymic epithelial cells (mTECs) have a primary role in the negative selection and, in this context, AIRE acts as a crucial transcriptional regulator. In mTECs, AIRE induces promiscuous gene expression (pGE) of tissue-specific antigens (TSAs), which are, then, presented to maturing T cells. Autoreactive T cells that recognize these TSAs with high affinity undergo negative selection through their apoptosis or, alternatively, regulatory T cells (Treg) are generated in order to prevent autoimmunity (2, 3).

Autoimmune regulator gene encodes a 545 amino acid protein with a molecular weight of 58 kDa (1). Starting from the amino terminus, AIRE is composed of a caspase recruitment domain (CARD)/homogeneously staining region (HSR), nuclear localization sequences (NLS), a SAND (Sp100, AIRE NucP41/75, and DEAF) domain, two planthomeodomain (PHD) zinc fingers, a proline-rich region (PRR), and four LXXLL motifs (where L stays for leucine) distributed among the domains (4). The CARD/HSR is involved in the process of AIRE homomultimerization and seems also to anchor AIRE to the chromatin (4, 5). The NLS has a stretch of basic amino acids at positions 131–133 important for nuclear import (4). The SAND domain does not have a distinct DNA-binding motif, but it is involved in promoting a protein–protein interaction with a transcriptional repressive complex (6). The two AIRE PHD fingers form a structural system for the recruitment of chromatin-related proteins and are engaged in AIRE transcriptional activity (7–9). LXXLL motif and PRR are implicated in promoting gene transcription (4).

Autoimmune regulator has a strict spatiotemporal regulation, being ubiquitously transcribed during the earliest stages of embryogenesis, and then restricted to thymic cells (mTECs and B cells) and extra thymic hematopoietic stem cells that may have a role in CD4 tolerization (10, 11).

At the transcriptional level, the expression of AIRE in mTECs and peripheral lymphoid organs is regulated by receptor activator of nuclear factor κB (RANK) signaling and therefore by nuclear factor κB (NF-κB)-induced transcription through an upstream conserved non-coding sequences (CNSs) of the *Aire* gene containing NF-κB-binding sites (12, 13). In addition, post-transcriptional mechanisms seem to modulate AIRE expression. A dioxygenase that catalyzes lysyl hydroxylation of splicing regulatory proteins (Jmjd6) is critical for AIRE expression. In fact, the intron 2 of *Aire* gene is not effectively spliced out in the absence of Jmjd6, resulting in marked reduction of mature Aire protein in mTECs and spontaneous development of multi-organ autoimmunity in mice (14).

The AIRE protein resides inside the nucleus, where it exhibits a speckled localization pattern (15). AIRE is a key regulator of TSA expression in mTECs and affects the transcription of thousands of TSA genes in a "stochastic" and "ordered" manner (16, 17). Indeed, a small percentage (1–3%) of the total number of mTECs expresses a particular TSA (18). Different sets of TSAs are regulated by AIRE within individual mTECs but whether a particular AIRE-regulated TSA is expressed in a given mTEC seems to be highly probabilistic (18, 19). Moreover, the "ordered" TSA expression refers to the increased likelihood that a particular set of TSA genes will be coexpressed in an individual mTEC (20) (**Figure 1**).

Autoimmune regulator acts in a very unusual way among transcription regulators, as it has no clear DNA-binding motif but seems to recognize genes that possess silenced chromatin states (6, 8, 9, 16). AIRE does not directly initiate TSA gene transcription, but it promotes TSA expression through the release of stalled RNA polymerase, RNA elongation, and splicing of target TSAs (15, 21). Moreover, AIRE binds to several partners that have the potential for post-translational protein modification, including the modification of AIRE itself and that seem to be critical for its biological function (22–24).

Recent insights on AIRE's regulation come from experimental studies which suggest that estrogen induces epigenetic changes in the AIRE gene, leading to reduced AIRE expression

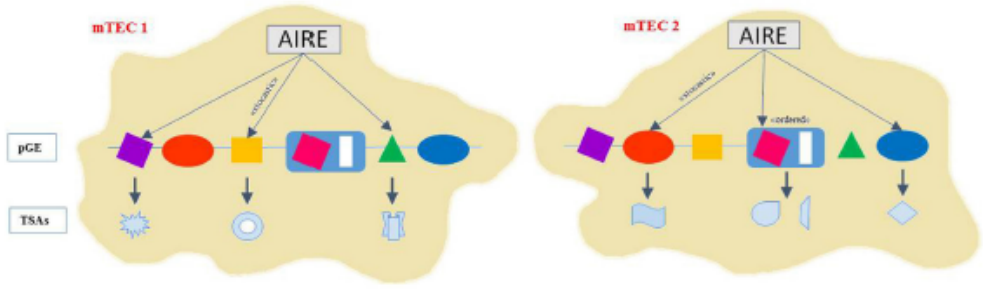


**FIGURE 1 | AIRE controls gene expression with ordered stochasticity.** AIRE seems to regulate pGE in mTECs in an apparently stochastic manner. Thus, single mTECs would express TRAs of mixed tissue origin rather than emulating cell line age-affiliated patterns displaying the highest degree and diversity of pGE. Indeed, different sets of TSAs are expressed in mTECs but whether a particular AIRE-regulated TSA is expressed in a given mTEC seems to be highly probabilistic. The "ordered" TSA expression refers to the increased likelihood that a particular set of TSA genes will be coexpressed in an individual mTEC. Coexpressed gene loci tend to colocalize to the same nuclear subdomain and TSA subsets align along progressive differentiation stages within the mature mTEC subset.

under a threshold that increases susceptibility to autoimmune diseases (25).

In summary, induction of pGE by AIRE is dependent on a complex regulatory mechanism which has only been partially unraveled so far.

In addition to the key role exerted on pGE, AIRE seems also to be critical for thymic generation of Treg cells during the perinatal period (3, 26). However, on this issue, further work is needed (3).

Moreover, recently a new hypothesis on Aire functioning in tolerance has been postulated. Aire may enforce immune tolerance by ensuring that autoreactive T cells differentiate into the Treg cell lineage; dysregulation of this process results in the diversion of Treg cell-biased clonotypes into pathogenic conventional T cells (27).

Furthermore, AIRE has several functions that are independent of its promotion of TSA expression in mTECs such as immunoregulatory functions in extrathymic AIRE-expressing cells and thymic B cells (15, 28). Moreover, AIRE enhances negative selection by regulating the repertoire of thymic dendritic cells and promoting apoptosis of mTECs (29, 30).

Finally, it has been postulated that AIRE regulates thymic maturation and architecture, probably through the expression of microRNAs (15, 31–34).

In summary, although our knowledge has increased in recent years, we still lack a coherent model incorporating and explaining all the intricacies of AIRE and its role in the regulation of immunological tolerance.

## NEW INSIGHTS INTO AIRE MUTATIONS

In humans, AIRE, identified on chromosome 21q22.3 by positional cloning in 1997, consists of 14 exons spanning 11.9 kb of genomic DNA (15). Mutations in the AIRE gene result in the development of APECED, a rare autoimmune condition, but reported worldwide, with a higher prevalence in genetically isolated populations (1).

Nowadays, 101 APECED-causing mutations have been found throughout AIRE (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=AIRE). These mutations include nonsense/missense mutations, deletions, or insertions and often abolish AIRE transcriptional activity or its localization to nuclear bodies (15, 35).

Despite its monogenic nature, APECED is characterized by a wide variability of the clinical expression and no strong genotype–phenotype correlation has been found among several populations (1, 35). Noteworthy, this lack is exemplified by the significant intrafamilial differences even between siblings carrying the same mutation, suggesting that disease-modifying genes, environmental factors, and immune system dynamics may play a role in modulating clinical expression of the syndrome (36, 37).

Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy has been originally considered an autosomal–recessive disease, and most mutations were assumed to be inherited in an autosomal–recessive manner, except for one mutation in the SAND domain, p.G228W, which exerts a dominant inheritance pattern (38).

However, recent evidences highlight that also heterozygous mutations of the gene can be associated with increased susceptibility to autoimmune diseases or incomplete forms of APS-1 (39). Patients with atypical or incomplete manifestations of APECED or with other immune diseases carrying heterozygous mutations of AIRE have been also described (38, 40–49). Cervato et al. showed different AIRE mutations in heterozygous state in relatives of APECED patients with various degrees of autoimmune or non-autoimmune diseases, but none of which affected by one of the major components of APECED (50).

Recently, Oftedal et al. reported a group of novel monoallelic and dominant-negative AIRE mutations clustered within the first PHD1 zinc finger domain in patients with various degrees of autoimmunity (39). The PHD1 domain is critical for AIRE's transcription–transactivation activity and mutations in this domain seems to affect the structure and thus the function of the entire AIRE tetramer. However, the significance of these monoallelic mutations is still unclear since the same alterations were found in varying autoimmune phenotypes, ranging from milder phenotypes of late-onset APECED to autoimmune polyglandular syndrome type 2 (APS-2) and isolated organ-specific autoimmunity following incomplete inheritance. A possible explanation is that AIRE tetramers still have some residual activity sufficient to ensure partial self-tolerance. Moreover, PHD1 mutations scanned in a public databases revealed an estimated frequency of about 0.0008, which is in the range of several autoimmune conditions that affect about 1 in 1,000 people, thus suggesting that mutations in AIRE might be more widespread in patients with autoimmunity than previously thought (39).

Moreover, Sparks et al. identified additional dominant-negative AIRE mutations associated with the modulation of insulin gene expression in thymus which is essential to induce either insulin tolerance or the development of insulin autoimmunity and type 1 diabetes (51).

## APECED: FROM "CLASSICAL" TO "NON-CLASSICAL" PHENOTYPE

In the light of these new knowledges, the original classification of APECED as unique autosomal–recessive disease seems to be incomplete. Taking into account the huge spectrum of phenotypes related to AIRE mutations, Oftedal et al. interestingly proposed to differentiate APECED in two major forms: (1) "classical APECED," characterized by recessive inheritance, presence of at least two of the three main components, and interferon (IFN) antibodies; and (2) "non-classical APECED," characterized by dominant heterozygous mutations mainly in AIRE's PHD1 zinc finger and a milder, less penetrant autoimmune phenotype (39) (**Table 1**).

Classical diagnosis of APECED has been originally defined by the presence of two of the three most common features: chronic mucocutaneous candidiasis (CMC), chronic hypoparathyroidism (CH), and Addison's disease (AD) (52).

Neutralizing autoantibodies against type 1 IFN (especially IFN-ω and IFN-α) have been found to strictly correlate with AIRE deficiency, thus leading to consider these autoantibodies as a precocious diagnostic tool for APECED and an additional diagnostic criteria for the diagnosis of APECED (52, 53). However, IFN

**TABLE 1 | APECED in "classical" and "non-classical" forms.**

| | "Classical APECED" | "Non-classical APECED" |
|---|---|---|
| Inheritance | AR | AD |
| Mutation | Homozygous/ compound heterozygous | Heterozygous |
| Phenotype | APECED (two of the three main components) | Various degrees of autoimmunity (from late-onset classical APECED or APS-2 to isolated organ-specific autoimmunity, i.e., vitamin B12 deficiency, pernicious anemia, vitiligo) |
| Onset | Childhood | Childhood/adulthood |
| Penetrance | Complete | Incomplete |
| IFN antibodies | Present | Variable |

autoantibodies seem to be less prevalent in the "non-classical" form, probably reflecting some residual AIRE function (39).

Molecular analysis of *Aire* may help to confirm the clinical diagnosis, especially in those cases with an atypical presentation.

Both "classical" and "non-classical" phenotypes are characterized by a wide heterogeneity in the severity and in the number of components among affected subjects with a wide variability even between siblings with the same genotype (39, 54).

Chronic mucocutaneous candidiasis is the first sign to appear followed by CH, before the age of 10 years, and later by adrenal insufficiency. However, a precise chronological order is not always present (52).

In addition to the classic triad (CMC, CH, and AD), the phenotype of APECED includes several autoimmune manifestations, which in some cases may also precede the classical triad (52).

The spectrum of endocrinopathies associated with APECED includes hypergonadotropic hypogonadism, type 1 diabetes (T1D), autoimmune thyroid diseases (ATD), growth hormone (GH) deficiency, and other pituitary defects (52).

The appearance of ectodermal abnormalities is also quite common including dental enamel hypoplasia, pitted nail dystrophy, and alopecia. Keratopathy, vitiligo, calcifications of the tympanic membranes, and periodic maculopapular, morbilliform, or urticarial rash with fever (52) are also included in the clinical spectrum of APECED.

Furthermore, gastrointestinal autoimmunity in APECED may lead to autoimmune gastritis, autoimmune hepatitis (AIH), intestinal disorders with chronic diarrhea alternating with obstipation, and cholelitiasis (54).

Asplenia, tubulointerstitial nephritis, interstistial lung disease (ILD), vasculitis, Sjogren's syndrome, cutaneous vasculitis, hemolytic anemia, scleroderma, metaphyseal dysplasia, and celiac disease have also been reported in APECED (55–58). Recently, a diagnosis of APECED was established by performing whole exome sequencing in a patient with increased renal echogenicity on renal ultrasound (59).

Muscle disease, with very similar clinical features of progressive limb-girdle myopathy, is a rare component of APECED (60).

To date, two patients with APECED have been affected by encephalitis leading to a severe and life-threatening condition (61, 62).

The "non-classical" form of APECED has been suggested to be characterized by variable autoimmune phenotypes, ranging from late-onset APECED to different combinations of autoimmune manifestations (APS-2), isolated organ-specific autoimmunity or autoantibodies, but no signs of autoimmune disease within individuals who harbor monoallelic AIRE mutations. In particular, families with vitamin B12 deficiency, pernicious anemia, and/or vitiligo at early age have been found to carry heterozygous PHD1 mutations, although the clinical phenotype has been expanded when larger materials were investigated. Indeed, organ-specific autoimmunity in the heterozygous cases seems to present in milder form and incomplete penetrance with respect to classical (39). These observations open a new window on the possibility that mutation carriers have a risk for developing some degree of APECED or other form of polyendocrinopathy.

However, more research is needed to determine the contributions of such AIRE variants to autoimmune susceptibility, especially in kindreds with a strong family history of autoimmunity.

In either "classical" or "non-classical" form of APECED, early diagnosis and regular surveillance, including periodic evaluation of hormonal and biochemical parameters, are essential to allow the prevention of severe and life-threatening events (i.e., hypocalcemia, adrenal crisis), even in the absence of clinical symptoms (63).

## OLD AND NEW AUTOANTIBODIES

Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy features multi-organ autoimmunity and autoantibody responses against target molecules with restricted tissue expression profiles. Consequently, autoantibody markers have acquired a central role in research and clinical diagnosis of APECED, providing a tool for diagnosis, and as predicting factor for the clinical course of the disease.

Chronic mucocutaneous candidiasis is a sign of the underlying immunodeficiency. Although the pathogenesis of CMC seems to be different from the all other autoimmune manifestations of the disease, an autoimmune pathogenesis for APECED-related CMC has also been proposed (64). APECED patients also develop high titer of neutralizing autoantibodies against IL-22, IL-17F, and IL-17A (64). Indeed, the neutralizing autoantibodies to Th17 cytokines or the impaired production of IL-22 and IL-17A seem to be associated with susceptibility to *Candida* infection (64).

The occurrence of endocrine manifestations is usually associated with a specific array of organ-specific autoantibodies. NATCH leucine-rich repeat protein 5 (NALP5) has been identified as the target for autoimmune attack in the parathyroid cells (65) in APECED. Autoantibodies specific for the steroidogenic enzymes (CYP21A2 and CYP17A1) and side chain cleavage enzyme (CYP11A1) are useful markers for the autoimmune destruction of the adrenal cortex even years before the clinical onset of the disease (5, 66). Autoantibodies to cytochrome CYP11A1 are associated with ovarian insufficiency (66). T1D is correlated with autoantibodies against insulin, IA-2 tyrosine phosphatase-like protein, and glutamic acid decarboxylase GAD65 (67).

Autoimmune hepatitis is characterized by the presence of autoantibodies specific for liver-expressed cytochromes CYP1A2 and CYP2A6 (67). Gastrointestinal symptoms have been associated with the presence of autoantibodies against tryptophan hydroxylase (TPH) (68). Enteroendocrine cells can also be the target of an autoimmune attack. Therefore, in some cases, the intestinal dysfunction might be viewed as an autoimmune endocrinopathy (69). Recently, circulating autoantibodies to Paneth cell-specific alpha 5 defensin and reduced numbers of Paneth cells in APECED patients have been reported and also associated with intestinal dysfunction (70).

Autoantibodies against TPH have been associated with alopecia, vitiligo, and enamel dysplasia and anti-SOX9/SOX10 antibodies with vitiligo (71, 72).

Autoantibodies directed against the potassium channel regulatory protein (KCNRG) and BPIFB1, found in epithelial cells of terminal bronchioles, have been suggested as a marker for pulmonary disease in APECED patients (64, 73).

The autoimmune nature of renal destruction has been confirmed by examining biopsy samples and by determining antiproximal tubular autoantibodies (74, 75). Furthermore, autoantibodies targeting kidney collecting ducts specific antigens [aquaporin 2 (AQP2) and two transcription factors regulating the aquaporin 2 promoter, namely homolog of the human homeobox B7 (HOXB7) and NF of activated T cells 5 (NFAT5)], have been recently identified in APECED patients affected with tubulointerstitial nephritis (76).

Recently, B cell response against a panel of over 9,000 human proteins has enabled to have a detailed profiling of known autoantigens and to identify novel immune targets in APECED. As for the lack of genotype–phenotype relationship, it has been shown that AIRE genotype did not appear to be an important determinant of autoantibody expression. Moreover, two novel gonadal autoantigens, melanoma antigen family B 2 (MAGEB2) and protein disulfide isomerase-like testis (PDILT), have been identified that potentially could contribute to infertility in male and female patients with APECED (77). Another mechanism proposed to explain subfertility in males with APECED is the presence of autoantibodies against the prostatic antigen transglutaminase 4 (TGM4), causing prostatitis, and possible abnormal sperm maturation (78).

Neutralizing autoantibodies specific for type I IFNs discovered in 2006 by Meager et al. (79) are hallmark of the "classical" APECED and are detectable in AIRE-deficient children as early as a few months of age, before the appearance of clinical symptoms or organ-specific autoantibodies (79). Autoantibodies against IFN seems to be less prevalent in "non-classical" APECED, probably reflecting some residual AIRE function (39).

In conclusion, we should take into account that the autoimmune response in APECED appears orders of magnitude more limited than could be expected. There is a great discrepancy between the number of AIRE-controlled genes (around 4,000) and the number of detected autoantigens in APECED (around 20). Several explanations must be considered. First, it could be that only a subset of self-antigens is able to activate autoimmune responses. Moreover, the peripheral tolerance mechanisms may provide additional filters for the development of autoimmunity. Finally, it has been observed that autoimmunity in APECED preferentially targets molecules with restricted tissue expression profiles.

## NEW INSIGHTS INTO AIRE GENETICS AND FUNCTIONING: CLINICAL IMPLICATIONS

The genetic basis of autoimmunity is a complex problem. The main lesson from recent evidence is that the mutations of AIRE can lead to various degrees of clinical autoimmunity, ranging from "classical" APECED to specific autoimmune conditions, which had not been previously mined for genetically determined conditions. Therefore, partial alterations of AIRE could play a role in common autoimmune disease; however, to measure the penetrance and the relative risk conferred by pathogenic AIRE mutations in its monoallelic variants, it will be necessary to sequence *Aire* in large cohorts of healthy individuals and autoimmune patients and to characterize experimentally in-depth all mutant alleles.

Furthermore, in the last decade, knowledge of AIRE's function and regulation has been significantly expanded leading to the identification of several partners and regulators of AIRE. Taken together, these molecular insights open new perspectives in understanding the phenotypic variability related to AIRE mutations and might provide interesting targets for novel therapeutic approach.

Indeed, an unanimously accepted effective therapy for APECED is not currently available. The use of immunosuppressive treatment in this category of patients may lead to a transient immunodeficiency with the risk to worsen their CMC and seems not able to stop the progression of all APECED manifestations (80). Thus, the management is mainly based on the care of each individual component and is mainly characterized by substitutive treatments for hormone deficiencies and immunomodulators have been only used in selected severe phenotypes (80, 81).

Although thymic transplantation has been proven useful in the treatment of differentiative thymic disorder (82), no data are available on this intervention on alterations of the thymic negative selection process. Thymic compartment can be targeted to modulate immune tolerance, for example, by enhancing AIRE expression, promoting deletion of self-reactive T cells and enhancing positive Treg cell selection, or inducing differentiation of TECs from pluripotent stem cells, offering new exciting possibility in therapeutic manipulation (15).

In conclusions, the new insights in the biology of AIRE and its control in immune tolerance offer exciting possibilities for the exploration of diagnostic and therapeutic strategies that would benefit APECED patients.

## AUTHOR CONTRIBUTIONS

All the authors contributed equally to this work.

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# Congenital absence of portal vein system and nodular regenerative hyperplasia (NHR) in a patient with incontinentia pigmenti: expanding the spectrum of clinical manifestations associated with alterations of the IKBKG/NEMO locus



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# Congenital absence of portal vein system and nodular regenerative hyperplasia (NHR) in a patient with incontinentia pigmenti: expanding the spectrum of clinical manifestations associated with alterations of the IKBKG/NEMO locus

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Summary
Incontinentia Pigmenti (IP) is a rare disease caused by mutations of NF-kB essential modulator (NEMO)/IkB kinase-c (IKBKG) gene, characterized by abnormalities of eyes, central nervous system, skin and cutaneous annexes. We describe the case of a patient affected with IP in which a congenital absence of portal vein system and nodular regenerative hyperplasia of the liver were detected. Microvascular impairment has already been reported to be involved in the retinal lesions and in the neurological symptoms observed in the disease. This firstly reported malformation broadens the spectrum of clinical manifestations associated with alterations of the IKBKG/NEMO locus and serves as new evidence supporting the role of NEMO in the development of the vascular system.

What's already known about this topic?
• Incontinentia pigmenti (IP) is a rare X-linked dominant disorder due to mutations in IKBKG/NEMO affecting skin, eyes and central nervous system. Microvascular alterations involving retina, central nervous system and lung have been described in some patients.

What does this study add?

• Congenital absence of portal vein system and nodular regenerative hyperplasia (NHR) observed in a patient with IP may share a microvascular pathogenesis with already reported retinal and neurological manifestations suggesting a role of IKBKG/NEMO pathway in the development of vascular system.

Incontinentia Pigmenti (IP) is a rare X-linked genodermatosis, characterized by skin, hair, teeth, nails, eyes and central nervous system (CNS) alterations, due to mutations of NEMO/IKBKG gene. The diagnosis is based on clinical criteria and confirmed by the molecular analysis. Patients usually present with characteristic skin lesions, which evolve through four stages (blistering, wart-like rash, swirling macular hyperpigmentation and linear hypopigmentation). Ectodermal alterations are a common finding and include alopecia, oligodontia, abnormal tooth shape and nail dystrophy. Approximately 30% of the patients show CNS alterations leading to the development of seizures, cognitive delays and learning disability. Patients with IP also exhibit an increased risk of developing retinal detachment due to neovascularization of the retina. Other vascular alterations have been reported including fatal pulmonary hypertension, due to microvascular abnormalities in the lung, peripheral artery disease and cerebral arteriopathy. [1, 2, 3, 4, 5, 6] Cardiac abnormalities, including tricuspid insufficiency and pulmonary vein-to-superior vena cava shunt, have been described in some cases. [7]

Here, we report on the case of a patient with IP in which a congenital absence of portal vein system (CAPVS) and nodular regenerative hyperplasia (NRH) of the liver were identified. This finding broadens the spectrum of clinical manifestations associated with alterations of the IKBKG/NEMO locus and provides new evidence supporting the role of NEMO in the development and in the homeostasis of the vascular system.

Case report

A 10-year-old female was born at term to healthy non consanguineous parents from an uncomplicated pregnancy. At the age of 45 days, the diagnosis of IP was suspected on the basis of persistent skin eczema and of the histological examination of skin sample, that revealed marked epidermal spongiosis, dyskeratotic keratinocytes, outbreaks of intra-epidermal keratinization, eosinophilic infiltrate in the papillary dermis.

The molecular analysis by PCR of NEMO gene revealed a deletion of exons 4-10 not present in the parents, while the array-CGH showed the presence of a duplication of the region 11q25, including the Neurotrimin (NTM) gene and a deletion of the region 14q32.33, including a part of the immunoglobulin heavy locus (IGH) gene and of the region 15q11.2, which includes the Non Imprinted In Prader Willi/Angelman1 (NIPA1) and NIPA2 genes inherited from the asymptomatic parents. Other clinical features included mild mental retardation associated with the evidence of perivascular gliosis at the cerebral magnetic resonance imaging (MRI), hyperintensity of the globus pallidum on T1-weighted images and an enlargement of the pericerebellar and occipitoparietal subaracnoid spaces.

At the age of 9 years, the abdominal ultrasound revealed the presence of a hyperechogenic liver lesion, measuring about 27 mm, suggestive of a hepatic hemangioma. A MRI using T1- and T2-weighted imaging and paramagnetic contrast enhancement was performed to better clarify the nature of the lesion: it revealed the presence of liver enlargement and multiple (> 10) not vascularized hepatic lesions, hyperintense on T1-weighted images and with low T2 signal, located in all the segments of the right lobe, interpreted as NRH. The injection of the contrast medium revealed the absence of the portal vein system, the presence of a large extrahepatic shunt (maximum diameter 16 mm) originating from the superior mesenteric vein and an ectasia of the inferior cava vein (ICV) (Fig.1). To better define the morphology of the portal vein system and to evaluate a therapeutic approach an angio-computed tomography (CT) scan was performed. A small vessel of 5 mm of diameter was identified at the portal confluence.

The diameter of this vessel progressively reduced approaching the ICV and was not possible to characterize the site of connection between the vessel and the ICV. Both the superior and the inferior mesenteric veins were ectasic and together with the splenic vein drained to the systemic circulation through a large group of collateral circles, flowing into the hemorrhoidal plexus and the internal iliac vein (Fig.2-3).

Liver function was evaluated and was only slightly impaired (AST 58 U/L, ALT 48 U/L, GGT 66 U/L, Serum Albumin 3.9 g/dl, PT-INR 1.39, APTT 43 sec). A moderate increase of the ammonium levels was identified. Esophago-gastro-duodenoscopy was performed and oesophageal varices were excluded. Since CAPVS may be associated with cardiac alterations, cardiac ultrasound was performed and revealed a mild tricuspid insufficiency.

As far as therapeutic options are concerned, no surgical procedure was recommended, considering the overall good clinical conditions. Nevertheless, taking into account cancer risk (haepatocarcinoma, haepatoblastoma) an intensive sonographic follow-up was scheduled, along with clinical and laboratoristic follow up in order to monitor liver function and exclude the development of hypersplenism.

## Discussion

IP is a rare genodermatosis, inherited as dominant X-linked trait, associated with *in utero* death of the affected males. Although the cutaneous lesions represent the hallmark of the disease, leading to the clinical diagnosis, many other developmental disorders of the neuroectodermal tissues may be identified. Alterations of the vascular system, affecting the CNS and the retina have already been reported in a low number of patients, but represent the most severe manifestations. Evidence suggests that the retinal lesions and the neurologic impairment develop as a result of vaso-occlusive events and ischemia with subsequent compensatory vasoproliferation. [8] In a recent study, Ridder et al. showed that the Transforming growth factor beta-activated kinase1 (TAK1)–NEMO signaling plays a key role in protecting the brain endothelium from the inflammatory injuries. The specific deletion of NEMO in brain endothelial cells of mice causes the disruption of the microvascular perfusion leading to the development of the neurological symptoms of IP.

In this case we reported on a patient affected with IP in which NRH and CAPVS have been identified. NRH may reflect an adaptive hyperplastic reaction of hepatocytes to different injuries, as microvascular changes involving the portal vein of the case herein reported. In this context, the pathogenesis of NRH is similar to the pathogenesis of retinal and CNS abnormalities already described in IP. Interestingly, male NEMO/IKKγ knockout mouse models die *in utero* because of massive liver apoptosis. [9,10,11]

In conclusion, our case report expands the spectrum of clinical manifestations of IP and highlights the role of the IKBKG/NEMO pathway in the development and in the homeostasis of the vascular system.

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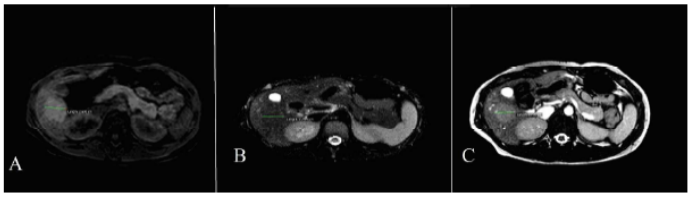


Fig.1 MRI Imaging of the abdomen, axial views. A) T1 weighted pre-contrastographic phase showing a spontaneously hyperintense nodule (green line), located in the V-VI segment, the biggest out of more than 10 detected in the right lobe. B) Fat-suppressed, T2-weighted image showing hypointense nodule. C) Balanced Turbo Field Echo sequence showing slightly hyperintense nodule.

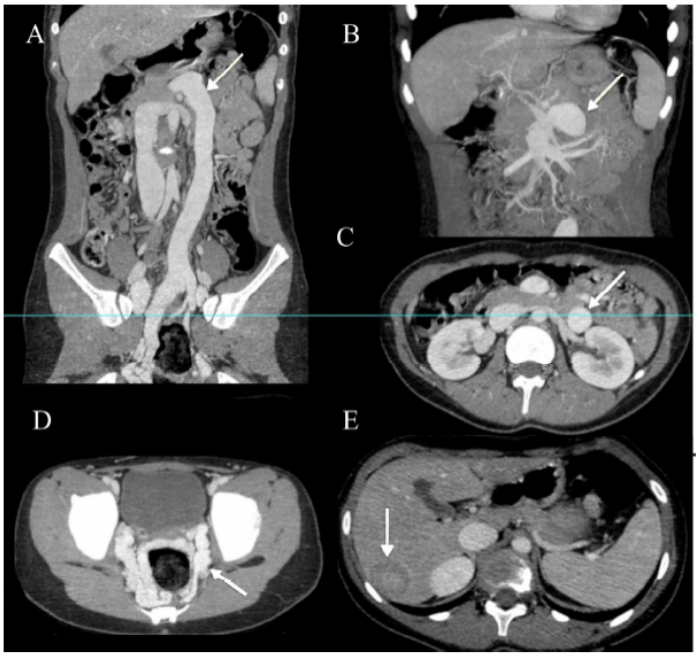


Fig.2 Contrast Enhanced Angio-Computed Tomography (CT) scan. A) Multi Planar Reformatted (MPR) Coronal view: extra-hepatic shunt (arrow) taking its origin from Superior Mesenteric Vein (SMV) and ectasic Inferior Vena Cava (IVC). B) Maximum Intensity Projection (MIP) Coronal view: confluence (arrow) of the extra-hepatic shunt with SMV. C), D) Axial views following the course (arrows) of the shunt in the abdomen and in the pelvis. E) Axial view: Nodular Regenerative Hyperplasia of the right lobe (arrow).

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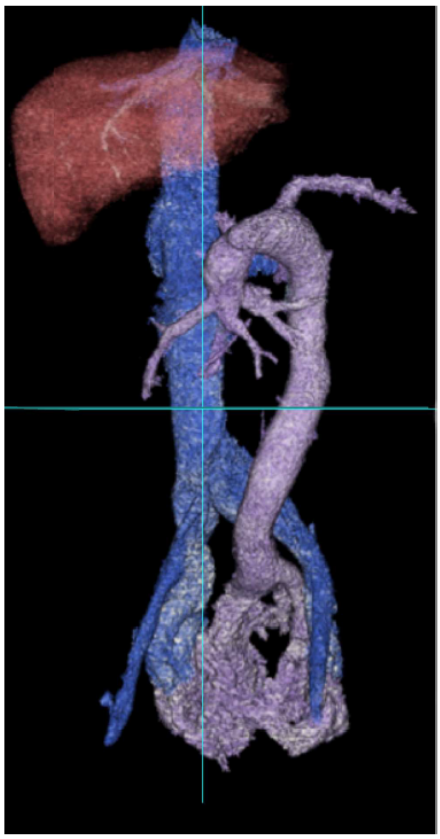

Fig.3 Contrast enhanced Angio-CT Scan. 3D Volume Rendering displaying supra-hepatic veins, Congenital Abscence of Portal Vein System (CAPVS) and extra-hepatic shunt originating from an ectasic SMV (arrow) and draining into a large group of collateral circles.

# CHAPTER VI

## "Phenotypic, Immunological and Molecular Characterization of New Forms of Primary Immunodeficiencies"

Early diagnosis of PIDs is crucial to establish a proper treatment and improve the overall outcome.

In the traditional approach to PIDs, the molecular diagnosis has long been based on the sequencing of multiple genes by the Sanger method, a time-consuming strategy, which often results in delayed diagnosis (86).

In the last years, next-generation sequencing (NGS) technology has become a valuable first-line diagnostic tool for the diagnosis of genetic disorders, in particular for conditions characterized by a very complex clinical presentation, revolutionizing the analysis of the human genome and its impact on health and disease.

NGS involves the parallel sequencing of hundreds of millions of DNA molecules. Advantages of NGS include the unbiased sequencing of the entire genome or exome at high depth of coverage. While whole genome sequencing (WGS) will report on the entire human genome, including exons, introns, regulatory regions and intergenic regions, whole exome sequencing (WES) is limited to the coding regions and splice junctions of the genome, which despite accounting for only ~2% of the genome, contain about 85% of genetic alterations known as responsible for human diseases. In PIDs, a targeted sequencing approach, restricted only to specific genes or to specific regions of interest is a reasonable possibility to identify putative pathogenic variants that explain a specific disorder. This can be the first-line alternative as it involves a smaller dataset than WES or (WGS) (87-92).

. Despite the technical and clinical advancements made thanks to the use of NGS, the identification of genetic defects in PIDs is still a major challenge. These challenges are of a technical as well as disease-inherent nature. Furthermore, not all PIDs are monogenic defects. For example, the most common form of antibody deficiency, common variable immunodeficiency disorder (CVID), is frequently a heterogeneous polygenic disorder.

Most autosomal dominant (AD) PIDs are caused by loss of function (LOF) alleles. Both null (complete lack of function) or hypomorphic (residual function, requiring residual expression of the gene product), have been described. Interestingly, since 2003, 17 AD disorders have been shown to be caused by GOF alleles. In theory, GOF alleles can be hypermorphic (increase in normal function) or

neomorphic (acquisition of a new function). Recently mutations of STAT1 gene have been shown to cause autosomal recessive (AR) PIDs by LOF and AD PIDs by LOF or GOF (11).

STAT1 GOF mutations are considered responsible for very complex and variable phenotypes, characterized by susceptibility to herpetic and fungal infections, autoimmunity, enteropathy, cardiac and vascular alterations, bronchiectasis, parodontitis and failure to thrive (93-94).

In the original article published in *Frontiers in Immunology* we retrospectively analized genetic variants identified through NGS technologies in patients with complex PIDs.

The description of the clinical and immunological phenotype of a patient with a STAT1 GOF mutation has been accepted for publication as Letter to the Editor on *Pediatric Allergy and Immunology.*

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ORIGINAL RESEARCH
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# Diagnostics of Primary Immunodeficiencies through Next-Generation Sequencing

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**Background:** Recently, a growing number of novel genetic defects underlying primary immunodeficiencies (PIDs) have been identified, increasing the number of PID up to more than 250 well-defined forms. Next-generation sequencing (NGS) technologies and proper filtering strategies greatly contributed to this rapid evolution, providing the possibility to rapidly and simultaneously analyze large numbers of genes or the whole exome.

**Objective:** To evaluate the role of targeted NGS and whole exome sequencing (WES) in the diagnosis of a case series, characterized by complex or atypical clinical features suggesting a PID, difficult to diagnose using the current diagnostic procedures.

**Methods:** We retrospectively analyzed genetic variants identified through targeted NGS or WES in 45 patients with complex PID of unknown etiology.

**Results:** Forty-seven variants were identified using targeted NGS, while 5 were identified using WES. Newly identified genetic variants were classified into four groups: (I) variations associated with a well-defined PID, (II) variations associated with atypical features of a well-defined PID, (III) functionally relevant variations potentially involved in the immunological features, and (IV) non-diagnostic genotype, in whom the link with phenotype is missing. We reached a conclusive genetic diagnosis in 7/45 patients (~16%). Among them, four patients presented with a typical well-defined PID. In the remaining three cases, mutations were associated with unexpected clinical features, expanding the phenotypic spectrum of typical PIDs. In addition, we identified 31 variants in 10 patients with complex phenotype, individually not causative *per se* of the disorder.

**Conclusion:** NGS technologies represent a cost-effective and rapid first-line genetic approach for the evaluation of complex PIDs. WES, despite a moderate higher cost

**Abbreviations:** ACMG, American College of Medical Genetics; ALPS, autoimmune lymphoproliferative syndrome; CMC, chronic mucocutaneous candidiasis; MYD88, myeloid differentiation factor 88; NGS, next-generation sequencing; PIDs, primary immunodeficiencies; SNVs, single nucleotide variants; TLR, toll-like receptor; T-NGS, targeted next-generation sequencing; WES, whole exome sequencing; WGS, whole genome sequencing.

compared to targeted, is emerging as a valuable tool to reach in a timely manner, a PID diagnosis with a considerable potential to draw genotype–phenotype correlation. Nevertheless, a large fraction of patients still remains without a conclusive diagnosis. In these patients, the sum of non-diagnostic variants might be proven informative in future studies with larger cohorts of patients.

**Keywords: primary immunodeficiencies, genetic diagnosis, targeted next-generation sequencing, whole exome sequencing, genotype–phenotype correlation**

## INTRODUCTION

Primary immunodeficiencies (PIDs) represent a heterogeneous group of monogenic disorders including more than 250 genetically defined diseases, mostly identified in recent years (1–3). A significant contribution to this rapid evolution is due to the integration of functional studies with the use of next-generation sequencing (NGS) technologies. The specific objective of this strategy is the identification of putative pathogenic alterations in known or novel genes implicated in well-defined biological pathways (4–7). However, despite the breadth of current knowledge, the diagnostic approach used for the identification of genetic causes underlying PID appears inefficient and time-consuming as the majority of PID cases remain without a diagnosis even after extensive clinical and genetic investigations (8). Moreover, even in a single PID gene mutation, a genotype–phenotype correlation is often missing, because various clinical phenotypes may be related to the same genetic defect and *vice versa* (9, 10). Nevertheless, early diagnosis in severe forms of PIDs is crucial to establish a proper treatment and improve the overall outcome (11, 12). In the traditional approach to PIDs, the molecular diagnosis has long been based on the sequencing of multiple genes by the Sanger method, a time-consuming strategy, which often results in delayed diagnosis (13).

Next-generation sequencing technology has become a valuable first-line diagnostic tool for the timely diagnosis of genetic disorders, in particular for complex clinical presentation (14). NGS allows rapid, cost-efficient, accurate, and high-throughput sequencing of millions of DNA fragments in a reasonably short time. Whole exome sequencing (WES) is limited to the coding regions and splice junctions of the genome, which despite accounting for only ~2% of the genome, contain about 85% of genetic alterations known as responsible for human diseases (15).

In PIDs, a targeted sequencing approach, restricted only to specific genes or to specific regions of interest is a reasonable possibility to identify putative pathogenic variants that explain a specific disorder, their related altered biological pathway, and the array of genes that are transcribed. This can be the first-line alternative as it involves a smaller dataset than WES or whole genome sequencing (WGS) and is easier for the management of the datasets (16–18). However, as the spectrum of distinct clinical entities and the presenting phenotypes are expanding because of the discovery of novel genes and of the identification of wider clinical phenotypes, the differential diagnosis and subsequent diagnostic targets must improve in parallel. This perspective makes the whole exome or genome sequencing strategy attractive options for the diagnosis of patients with PID (19).

Here, we report on a case series of 45 complex or atypical clinical phenotypes that were suggestive of PID, but difficult to diagnose in a timely manner using the current diagnostic procedures. Our aim was to use current NGS technology to help diagnose PID in this cohort of patients. We used T-NGS and WES in 27 and 18 patients, respectively.

## MATERIALS AND METHODS

### Patients

We have studied 45 patients with a clinical history highly suggestive of a primary immunological defect, that were heterogeneous for ethnic origin, age, and sex. The patients were selected on the basis of clinical features and abnormal immune parameters (20). The clinical criteria included one or more of the following features: opportunistic infections, granuloma, chronic mucocutaneous candidiasis (CMC), intractable diarrhea, bronchiectasis, and severe autoimmunity. These symptoms were associated, in some patients, with non-immunological features. In six patients, a positive family history for a similar phenotype was observed. Clinical criteria were considered if associated with one or more of the following quantitative and/or qualitative immunological abnormalities: abnormal lymphocyte subsets (absolute count <2 SD of normal values according to ESID criteria); proliferative response to mitogens <10% of the levels measured in the control subject; absent/poor specific antibody response; hypogammaglobulinemia; elevated IgE levels (>2000 kU/l); severe impairment of cytolytic activity; and alteration of class switch recombination (CSR) with or without hyper-IgM. PID patients, selected on the basis of the above specified criteria, were subsequently grouped on the basis of NGS results, functional alterations, and consistency between genotype, phenotype, and immune assays.

The study was approved by the Institutional Ethical Committee "Carlo Romano" of Federico II University and by Ethical Committee of Spedali civili (Brescia) and conducted after informed consent was obtained.

### DNA Extraction and Sequence Capture Array Design

Genomic DNA was isolated from peripheral blood lymphocytes with QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Quantity and quality were determined through the Epoch Microplate Spectrophotometer (BioTech Instruments, Winooski, VT, USA). A panel of 571 genes, including 68 genes known or predicted to be related to PIDs and/or immune regulation, was

sequenced (Table S1 in Supplementary Material). Basically, broad searches in literature, PubMed queries and expert suggestions defined the gene panel. We used BioMart (Ontario Institute for Cancer Research/European Bioinformatics Institute) to retrieve the coordinates of all exons for the specified genes from Ensembl. Coordinates were based on the current human reference genome (hGRC37, hg19).

## Next-Generation Sequencing and Bioinformatics Analysis

Samples were sequenced using either a targeted panel of 571 (TaGSCAN v2.0) or WES for enrichment. Briefly, TaGSCAN samples were prepared for sequencing using Illumina's TruSight Inherited Disease panel according to manufacturer's protocols (Illumina, San Diego, CA, USA). Samples were sequenced to at least 2.5 GB on an Illumina MiSeq with TruSeq MiSeq V3 reagents, yielding paired 250 nucleotide reads. Samples were prepared for exome sequencing using the TruSeq HT library preparation kit (Illumina; San Diego, CA, USA) followed by exome enrichment using the xGen Exome Research Panel V1.0 (Integrated DNA Technologies; Coralville, IA, USA) according to manufacturers' protocols. Paired-end 2 bp × 125 bp sequencing was completed on an Illumina HiSeq 2500 instrument in high output mode using V4 Chemistry. Samples were sequenced to at least 7 GB of data resulting in a minimum target coverage of 50×. For all samples, sequence was assembled, aligned to reference gene sequences based on human genome build GRCh37/UCSC hg19, and analyzed using custom-developed software, RUNES and VIKING (21).

Alignment, variant calling, and analysis were performed, as described previously (21). In detail, variants were filtered by frequency and variant category (22). Variant analysis was confined to coding and splice variants with a minor allele frequency (MAF) of 1% or less in the CMH internal database, in the Exome Variant Server (EVS)[1] and Exome Aggregation Consortium (ExAC).[2] Other tools used to restrict NGS analysis to a set of gene-associated regions relevant to clinical presentations, was symptom- and sign-assisted genome analysis (SSAGA), which mapped the clinical features in ill neonates and children to disease genes, and Phenomizer databases (21, 23). Sorting Intolerant From Tolerant (SIFT) and Polymorphism Phenotyping v2 (PolyPhen2) have been used to provide information on the impact of the variants.

## Functional Assays and Sanger Validation

Functional assays were performed to prove the impact of variants identified by NGS. Functional studies included lymphocyte proliferation assays performed through [$^3$H]thymidine incorporation assay, natural killer cell-mediated lysis of target cells and lymphocyte apoptosis induced by FAS death receptor cross-linking, evaluated through flow cytometry, cytokines production after toll-like receptors (TLR) stimulation, evaluated through real-time PCR. Furthermore, protein expression was evaluated through western blot analysis and standard procedures. After identification of genetic variants that were predicted to be damaging, along with consistent genotype–phenotype correlation, mutations were validated by Sanger sequencing using standard protocols. In 9 patients, we detected 11 variants that were confirmed by Sanger sequencing.

## Additional Phenotype-Based Variant Filtering Criteria

In order to identify potential causal mutations, annotated variants were further prioritized based on the following criteria:

- homozygous or heterozygous variants already reported that were related to any immunological clinical phenotype were selected to be further investigated by functional studies and validated by Sanger method;
- variants in genes implicated in a molecular pathway related to the phenotype were considered if associated with any functional alteration, which was even partially consistent, with the clinical phenotype;
- all the genetic variants of genes that were probably unrelated to the molecular pathway suspected to be involved in the pathogenesis of the disease were excluded from the functional studies, but reported in an *ad hoc* repository.

# RESULTS

## NGS of 45 Patients

Genomic DNA from 27 patients was enriched for all exons from 571 genes, including genes involved in immunological pathways, while DNA from 18 patients was enriched for all nucleotides of the exome. DNA from six patients, analyzed with TaGSCAN, was subsequently sequenced for the entire exome and the bioinformatic analysis of data results is currently ongoing. For TaGSCAN, 98.9% of base pairs targeted had at least 10× coverage. Mean depth of coverage was 580×. WES covered 97.05% of exonic regions at 10× or greater on average. The overall analytic sensitivity for single nucleotide variants (SNVs) was 98.7%.

The results of NGS exon sequencing are shown in **Tables 1–4**. After bioinformatics analysis and filtering, the potential causative nucleotide changes needed to be further evaluated in each individual patient (**Table 5**). All of these variants were SNVs or small indel variants, with no other alteration such as large deletions or insertions identified.

A satisfying molecular diagnosis of PID was achieved in 7 of the 45 patients (16%), including 3 patients with an atypical presentation. Three causative variations were identified through T-NGS, while five through WES. On the basis of the genetic findings and of the clinical phenotypes, patients were divided in four groups. Group 1 included subjects with a diagnostic genotype that was previously associated with an immunological and/or clinical phenotype already reported for that genetic disease. Group 2 included subjects with a diagnostic genotype associated with novel or atypical clinical features of a genetic disease. In the group 3, NGS revealed multiple genetic variants that were consistent only with some features of the clinical and immunological phenotype. Group 4 included patients with a complex unclassified disorder that was associated with multiple

[1] http://evs.gs.washington.edu/EVS.
[2] http://exac.broadinstitute.org/.

**TABLE 1 | Genetic variants associated with typical PID.**

| Patient | NGS method | Gene | Mutation | Protein | Zygosity | Inheritance | Clinical and immunological phenotype |
|---|---|---|---|---|---|---|---|
| 001 | T-NGS | *CD40LG* | c.373C>T | p.His125Tyr | Hom | XL | Severe hypogammaglobulinemia with hyper-IgM, neutropenia, *P. jirovecii* pneumonia, CMV infection, intractable diarrhea |
| 002 | WES | *STAT1* | c.847T>A | p.Leu283Met | Het | AD | Chronic mucocutaneous candidiasis, recurrent pneumonia, hypothyroidism, lymphopenia, poor vaccine response |
| 003 | WES | *BTK* | c.1105C>T | p.Leu369Phe | Hom | XL | Agammaglobulinemia |
| 004 | T-NGS | *JAK3* | c.856C>T | p.Gln286Ter | Hom | AR | $T^-B^+NK^-$SCID, chronic diarrhea, poor proliferative response to mitogens, IgA deficiency |

*CD40LG and JAK3 variants were identified with T-NGS and STAT1 and BTK variants identified with WES. These variations were found in patients with clinical phenotype of classic well-defined PIDs.*

**TABLE 2 | Genetic variants associated with novel features of PID.**

| Patient | NGS method | Gene | Mutation | Protein | Prediction score | | Zygosity | Inheritance | Clinical and immunological phenotype |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | SIFT | PolyPhen2 | | | |
| 005 | T-NGS | *MYD88* | c.192_194del | p.Glu66del | – | – | Hom | AR | Chronic yersiniosis and terminal ileitis, recurrent severe cutaneous granulomatous abscesses, hyper IgE, hypereosinophilia, neutropenia |
| 006 | WES | *PLDN* | c.232C>T | p.Q78X | – | – | Hom | AR | Partial oculocutaneous albinism, nystagmus, recurrent cutaneous infections, thrombocytopenia, leukopenia, NK deficiency |
| 007 | WES | *DOCK8/ CLEC7A* | c.3193delA | p.Ser1065Ala X17/p.Tyr238X | – | – | Hom/Hom | AR | Intractable diarrhea, eczema, malignancy, food allergies, hyper IgE, lymphopenia |

*MYD88 variant was identified through T-NGS in a patient with atypical features of MyD88 deficiency, presenting with chronic yersiniosis. PLDN variant was identified through WES in a patient with incomplete features of Hermansky–Pudlak type II syndrome and impairment of NK cytolytic activity. DOCK8/CLEC7A variants were identified for the first time in a patient with intractable diarrhea, malignancy, and features of Hyper IgE syndrome.*

**TABLE 3 | Genetic variants potentially involved in the immunological features.**

| Patient | NGS method | Gene | Mutation | Protein | Prediction score | | Zygosity | Major clinical features |
|---|---|---|---|---|---|---|---|---|
| | | | | | SIFT | PolyPhen2 | | |
| 8 | T-NGS | *UNC13D* | c.335G>C | p.Cys112Ser | 0.38 | 0.832 | Het | Acute lymphoblastic leukemia treated with allogeneic HSCT, ethmoiditis, recurrent lymphadenopathy, autoimmune cytopenia, arthritis, hypogammaglobulinemia, hyper-IgM, IgA deficiency |
| | | *CASP10* | c.683C>T | p.Pro228Leu | 0.07 | 0.071 | Het | |
| 9 | T-NGS | *CASP10* | c.1202_1208del | p.Cys401LeufsTer15 | – | – | Het | Alopecia universalis, hypothyrotropinemia, type I diabetes mellitus, dental enamel hypoplasia, developmental delay, short stature, candidiasis, hepatomegaly, multiple skeletal abnormalities, myopia, dysmorphic features, microcephaly, abnormal FAS-induced apoptosis. Hyper IgE |
| 10 | T-NGS | *DOCK8* | c.1907A>G | p.Lys636Arg | 0.4 | 0.057 | Het | Inflammatory bowel disease, short stature, aspergillosis, EBV infection, low $CD4^+$ lymphocyte subset, increased CD4 CD8 double-negative T cells, normal antibody response |
| | | *TLR3* | c.2672A>G | p.His891Arg | 0.01 | 0.309 | | |
| 11 | T-NGS | *ADA* | c.377C>A | p.Pro126Gln | 0 | 0.999 | Het | $T^-B^+NK^-$ SCID treated with bone marrow transplantation, *P. jirovecii* pneumonia, recurrent otitis, absent ossicular bone with hypoacusia of the right ear, mild brain, and cerebellar atrophy, speech delay, scoliosis |
| | | | c.1047A>G | p.K349= | – | – | | |
| | | *ERCC6* | c.3262A>G | p.Ser1088Gly | 0.48 | 0.001 | | |
| | | | c.2697G>A | p.T899= | – | – | | |
| 12 | T-NGS | *AP3B1* | c.787G>T | p.Gly263Cys | 0.05 | 0.932 | Het | Interstitial lung disease CMV infection, esophageal candidiasis, strabismus, abnormal expression of perforin in NK cells, reduction of $CD4^+$ cells with increase of $CD19^+$, normal proliferative response to mitogens, normal antibody response |
| | | *PRF1* | c.695G>A | p.Arg232His | 0.02 | 0.991 | | |
| | | | c.272C>T | p.Ala91Val | 0.01 | 0.808 | | |
| | | *ADAMTS13* | c.2701G>T | p.Ala901Ser | 0.61 | 0.049 | | |

*In this group of patients, T-NGS allowed to identify multiple variants not causative of a specific PID, but with a possible impact on the disease.*

**TABLE 4 | Genetic variants not causative of PID with undetermined impact on the disease.**

| Patient | NGS method | Gene | Mutation | Protein | Prediction score | | Zygosity | Major clinical features |
|---|---|---|---|---|---|---|---|---|
| | | | | | SIFT | PolyPhen2 | | |
| 13 | T-NGS | *CFTR* | c.2991G>C | p.Leu997Phe | 0.08 | 0.18 | Het | Late onset hypogammaglobulinemia, recurrent pneumonia, bronchiectasis, chronic sinusitis, cervical and mediastinal lymphadenopathy, recurrent abdominal pain, hepatomegaly with low grade steatosis, splenomegaly |
| 14 | T-NGS | *SYCE2* | c.577G>A | p.Val193Met | 0.28 | 0 | Het | t(11;18)MLT1-AP12 gastric maltoma HP+, persistent oral candidiasis, sinusitis; lung cystis, chronic cough, recurrent fever, hypereosinophilia, recurrent itch, recurrent myofascitis, hyper-IgM, altered somatic hypermutation, absent $CD19^+CD20^-$ $IgG^+$ (mature), low $CD19^+CD27^+IgM^+$ (memory), absent $CD19^+CD27^+IgM^-$ (switched memory) |
| | | *LYST* | c.10235G>A | p.Arg3412His | 0.02 | 0.997 | | |
| 15 | T-NGS | *ATR* | c.5257A>G | p.Ile1753Val | 0.16 | 0.403 | Het | Severe aplastic anemia, hepatomegaly, *Legionella* sp. and *Aspergillus* recurrent pneumonia, metacarpal deforming alterations with bone demineralization, abnormal lymphocyte proliferation, dilated cardiomyopathy, early retinopathy |
| | | *ARSA* | c.869G>A | p.Arg290His | 0.55 | 0.959 | Het | |
| | | *CASP10* | c.683C>T | p.Pro228Leu | 0.07 | 0.071 | Het | |
| | | *IKBIKG* | c.1165C>T | p.Pro389Ser | 0.29 | 0.041 | Het | |
| | | *MEFV* | c.460T>C | p.Ser154Pro | 0.04 | 0.001 | Het | |
| | | *SP110* | c.1114C>T | p.Arg372Ter | – | – | Het | |
| | | *UNC13D* | c.335G>C | p.Cys112Ser | 0.38 | 0.852 | Het | |
| 16 | T-NGS | *ATRX* | c.2247_2249del | p.Ser750del | – | – | Het | Autoimmune adrenal insufficiency, autoimmune thyroiditis, lymphadenopathy, autoimmune thrombocytopenia, and neutropenia |
| | | | c.2133_2135del | p.Ser712del | – | – | Hom | |
| | | *MYD88* | c.10_28del | p.Ala6ProfsTer39 | – | – | Het | |
| | | *DOCK8* | c.2920C>A | p.His974Asn | 0.34 | 0.001 | Het | |
| | | | c.3016C>A | p.His1006Asn | 0.33 | 0.003 | Het | |
| | | | c.3220C>A | p.His1074Asn | 0.33 | 0.003 | Het | |
| 17 | T-NGS | *TYK2* | c.3488A>G | p.Glu1163Gly | 0.27 | 0.006 | Het | Hypogammaglobulinemia, familial IgA deficiency, hyper IgE, multiple bronchiectasis, candidiasis |
| 18 | T-NGS | *TLR3* | c.634-10C>A intron | – | – | – | Het | Familial IgA deficiency, multiple bronchiectasis, recurrent respiratory infections, low IgM levels |
| 19 | T-NGS | *CASP10* | c.1094A>C | p.Tyr365Ser | 0 | 0.982 | Het | Mild hypogammaglobulinemia, undetectable $CD16^+$ lymphocyte levels, pervasive developmental disorder |
| | | *ERCC5* | c.2375C>T | p.Ala792Val | 0 | 1 | Het | |
| | | *GJC2* | c.256G>A | p.Val86Ile | 0.13 | 0.825 | Het | |
| | | *PEX26* | c.728C>T | p.Ala243Val | – | – | Het | |
| | | *RAB23* | c.536A>C | p.Glu 179Ala | – | – | Het | |
| | | *UBR1* | c.3290C>T | p.Thr1097Met | – | – | Het | |
| 20 | T-NGS | *CD3-ZETA* | c.301C>T | p.Gln101Ter | – | – | Het | Hypogammaglobulinemia, recurrent pneumonia, transient alopecia, behavioral disorders, oropharyngeal candidiasis |
| | | *OCRL* | c.2032A>G | p.Ser678Gly | 0 | 0.986 | Hem | |
| 21 | T-NGS | *PKHD* | c.5125C>T | p.Leu1709Phe | 0.15 | 1 | Het | Multi-organ failure and hypocalcemia during EBV infection, persistent EBV infection, kidney single cystic formation, hyper IgE, normal antibody response and proliferative response to mitogens, normal perforin intracytoplasmic expression, normal degranulation assay, reduced production of $IFN\gamma$ |
| | | *NPHP3* | c.2864del | p.Asp955ValfsTer2 | – | – | Het | |
| | | *G6PC* | c.634A>G | p.Ile212Val | 0.88 | 0.01 | Het | |
| 22 | T-NGS | *RAB27* | c.418C>G | p.Gln140Glu | 1 | 0.24 | Het | Recurrent fever, oral aphthous, diarrhea, laterocervical lymphadenopathy, hepatomegaly, increased level of amyloid protein, recurrent pneumonia, increased level of IgA, hyper IgE increased double-negative lymphocytes, normal functional Fas assay |
| | | *LYST* | c.6482A>C | p.Glu2161Ala | 0.03 | 0.002 | Het | |

*In this fourth group, T-NGS led to identify multiple genotypic alterations in patients showing complex phenotypes, which could not fit into defined clinical syndromes. In this group, no genotype–phenotype relationship was possible. However, since these cases are extremely rare, these data should likewise be collected in a database for future studies pointing to the sum of alterations on the whole.*

genetic variants, each of them, individually, was not *per se* causative of the disorder. However, regarding the variants of groups 3 and 4, it should be pointed out that, since parental cosegregation studies were not performed, the variants may not be truly disease-causing or disease-modifying alterations, especially if inherited together from an unaffected parent.

The first group included four subjects who have been identified for a genetic defect associated with a known well-established

**TABLE 5 | Gene variants confirmed by Sanger sequencing, functional assays and/or previous report.**

| Gene | Mutation | Sanger confirmation | Functional assays | Reference |
|---|---|---|---|---|
| *CD40LG* | c.373C>T | Yes | Absent expression of CD40L after stimulation | (24) |
| *BTK* | c.847T>A | Yes | NA | (25) |
| *STAT1* | c.1105C>T | Yes | IFNα- and IFNγ-induced increased level of pSTAT1 | (26) |
| *JAK3* | c.856C>T | Yes | Abnormal proliferative response to mitogens | – |
| *MYD88* | c.192_194del | Yes | Reduced levels of IL-6, IL-1, CCL2, and CCL3 after TLR stimulation with LPS, IL-1, TNFα; rescue of IL-1β and LPS responsiveness after WT *MYD88* gene transfection | (27, 28) |
| *PLDN* | c.232C>T | Yes | Absent PLDN protein expression | (29, 30) |
| *DOCK8/CLEC7A* | c.3193delA | Yes | Low level of DOCK8 protein expression | (31) |
| *UNC13D* | c.335G>C | NA | NA | (32) |
| *CASP10* | c.683C>T | NA | NA | – |
| *CASP10* | c.1202_1208del | Yes | Abnormal Fas-induced apoptosis in PHA-activated T cells | – |
| *DOCK8* | c.1907A>G | NA | – | – |
| *TLR3* | c.2672A>G | NA | Normal INFγ production after TLR3 stimulation | – |
| *ADA* | c.377C>A | NA | Abnormal proliferative response to mitogens | (33, 34) |
| *ERCC6* | c.3262A>G | NA | NA | – |
| *AP3B1* | c.787G>T | NA | NA | – |
| *PRF1* | c.695G>A | Yes | Reduced expression of perforin in NK cells | (35) |
| *ADAMTS13* | c.2701G>T | NA | NA | – |

*For each variant, the Sanger confirmation, the functional assays, and references for variants already published were reported.*
*NA, not applied.*

immunological and/or clinical phenotype of PID (**Table 1**). In detail, variants in *CD40LG*, *BTK*, *STAT1* genes were already reported in literature as pathogenic (24–26). The *JAK3* nonsense variant was predicted to result in nonsense-mediated decay resulting in no protein expression. This mutation, though not previously reported, was fully congruent with the classic JAK3–SCID phenotype observed in multiple affected family members who shared the same genotype (proband and two siblings).

The second group included other three subjects in whom genetic variants were found as associated with novel phenotypic features, in previously characterized phenotypes (**Table 2**). Specifically in this group, patient 005 was found to carry a homozygous mutation in *MYD88* (c.192_194del; p.Glu66del), previously reported in several MYD88 deficiency affected families (27, 28). This patient was of a Rom ethnicity and had inherited the deletion from his unaffected consanguineous parents. But, early infant deaths due to severe infections were observed in the same family pedigree. Functionally, known mutations result in impairment of cytokine production after TLR stimulation (36, 37). Patients 006 and 007 were found to carry homozygous deleterious mutations in *PLDN* and *DOCK8/CLEC7A* gene, respectively, whose phenotypic peculiarity has been described in detail (29, 31).

The third group of subjects included five patients who carried multiple heterozygous variants affecting genes expressed in the hematopoietic system that were not consistent with a specific PID (**Table 3**). However, some of these patients might harbor a second mutation that was not identified by NGS or Sanger sequencing. In patient 008, we observed a heterozygous, probably, pathogenic variant in *UNC13D* (c.335G>C p.Cys112Ser), and a heterozygous, most likely benign variant in *CASP10* gene (c.683C>T p.Pro228Leu; exon 10 not covered). This patient had a clinical phenotype consistent with an autosomal dominant type II ALPS (ALPS-II), associated with hypogammaglobulinemia and acute lymphoblastic leukemia. Even though the *CASP10* variant alone in the *in silico* prediction programs, PolyPhen2 and SIFT, was predicted to be tolerated and was observed in 35 healthy individuals (ExAC), its significance in association with the second deleterious variant in *UNC13D* has never been described and could be potentially relevant. In fact, this UNC13D variant has been previously reported in association with heterozygous mutation of *FAS* and is considered a disease modifier for ALPS (32). In patient 009, we found by T-NGS a heterozygous frameshift variant in *CASP10* causative of ALPS-II and categorized as likely pathogenic according to American College of Medical Genetics (ACMG) criteria (22). His immunological phenotype was characterized by very high IgE levels (>2000 IU). The functional analysis of Fas-induced apoptosis in PHA-activated T cells from the patient confirmed that the apoptotic pathway was impaired, since cell apoptosis upon triggering of Fas was impaired (92% survival; normal values: median 60%, 95th percentile 82%). His brother, who presented with similar manifestations, died early in life because of hemophagocytic lymphohistiocytosis. In addition, the patient 009 also had additional clinical features, such as developmental delay, microcephaly, peculiar facial dysmorphism, and skeletal abnormalities, which could not be at moment demonstrated or excluded to be directly explained by the variant. In patient 010, we found two heterozygous variants of unknown significance in *DOCK8* and *TLR3* with good coverage of each gene and no second variant. The *TLR3* variant had multiple lines of computational evidence supporting a deleterious effect on gene/protein. Further studies are ongoing to find a possible correlation between the association of the two variants with the clinical phenotype, which was characterized by inflammatory bowel disease, susceptibility to viral infections, aspergillosis, T-cell lymphopenia, and increased CD4 and CD8 double-negative T cells.

Alternatively, a second unidentified mutation of DOCK8 might account for the clinical manifestations of the patient. In patient 011 with a $T^-B^+NK^-$ SCID phenotype associated with deafness, microcephaly, brain and cerebellar atrophy, developmental delay, NGS revealed 2 heterozygous mutations of *ADA* gene, located in the same allele, the first one being likely pathogenic, and a further heterozygous variant of unknown significance of *ERCC6* gene. The latter gene, which encodes for Cockayne syndrome B protein, has been recently described as essential for postnatal neuronal differentiation and neuritogenesis (38). Intriguingly, the patient showed also some neurological manifestations, such as macrocephaly and moderate grade cerebral atrophy. In patient 012 affected with hemophagocytic lymphohistiocytosis and marked reduction of perforin expression in NK cells, we identified two heterozygous mutations in PRF1 that were located in the same allele as shown by Sanger sequencing. In the same patient, we have also detected heterozygous variants in AP3B1 and ADAMTS13 genes that have been implicated in NK activity as well.

The last group of patients included 10 patients with a complex disorder that was not typical of any known syndrome and was associated with multiple genetic variants, each of them was not *per se* causative individually of that disorder (**Table 4**). In this group, it was not possible to draw any correlation between the genetic variations that were detected and the pathogenesis of the disorders. However, these alterations of unknown biologic significance were found in several genes implicated in immunological functions at different extent.

In the remaining 23 patients who were analyzed by NGS, including 9 studied by T-NGS and 15 by WES, we could not identify any candidate variants. These unsolved datasets will be re-analyzed when new bioinformatics tools become available and as new disease genes are described.

## DISCUSSION

The advent of NGS technologies has given the possibility to physicians to investigate multiple genes assay, to provide great opportunities for diagnosing patients affected with complex disorders of the immune system, and to increase our knowledge on the pathogenesis of these genetic disorders (39).

In this study, we used NGS technologies to identify potential disease-causing mutations in patients affected with clinical phenotypes highly suggestive of a PID, which were still not diagnosed after using traditional sequential Sanger sequencing procedures. Thanks to this novel diagnostic approach, we report that a definitive diagnosis of PID was achieved in a timely manner in 7 out of the 45 subjects. In three patients, the diagnosis was achieved through T-NGS, while, in the other four patients, the diagnosis was reached by WES. In all these subjects, the application of a clear-cut filtering strategy, consisting of targeted sequencing, bioinformatics analysis, phenotype-based filtering criteria, and confirmartory functional assays and Sanger sequencing, led to the identification of the underlying immune disorder (**Figure 1**). With this approach, eight of the overall group of variants resulted in potentially disease-causing mutations, distributed over seven patients.

We have divided NGS results into four categories: (I) genetic alterations associated with a canonical PID phenotype, (II) diagnostic genotype in atypical presentation, (III) genetic variants potentially involved in the immunological features, and (IV) multiple genetic variants, each of them was not *per se* causative individually of the disorder, even though the sum of variations of the different genes could be proven in the future of some pathogenic significance, as either causative or modifier factor.

The first and second groups included patients whose candidate genotypes were congruent with the immunologic features suggesting a causal relationship. Of note, exome sequencing allowed the identification of *PLDN* variants associated with a novel genetic cause of partial albinism and with PID (29). This study reported six patients who had undergone a diagnostic odyssey of 10–21 months driven by worldwide accepted protocols (40). Unfortunately, this candidate gene approach, although functionally driven, failed to yield a diagnosis. The diagnosis was finally made possible with NGS technology in a much more timely manner (2 months).

In the second category of patients, which included patients carrying genetic variants previously reported, but associated with novel features of previously established phenotypes, the molecular definition of the diagnosis contributed to expanding the overall knowledge of pathogenetic mechanisms underlying that specific disorder. In the patient with MYD88 deficiency, the atypical presentation was characterized by chronic yersiniosis resulting in terminal ileitis and recurrent neutropenia, in the absence of invasive pneumococcal disease, as is expected in this rare immunodeficiency. The atypical clinical presentation was responsible for the diagnostic delay. Moreover, Sanger sequencing of four candidate genes (*STAT3*, *ELANE*, *RAG1*, and *RAG2*) had given negative results (27). Homozygous mutations of *DOCK8* and *CLEC7A* were already reported as genetic cause of two distinct PIDs, such as autosomal recessive Hyper IgE syndrome and of CMC, respectively, but never observed in a single patient. It is likely that these genetic variants affecting two distinct loci, and probably concurring to the clinical manifestations of the patient, could not be identified without the availability of NGS (31).

The cases highlighted herein contribute to the explanation of several phenotypes for very rare disorders. Such broadening of the phenotypic spectrum is a phenomenon shared with many other rare disorders, as atypical patients are identified through NGS. In the case of congenital immune disorders, at the beginning only the most severe forms are described (41). Only after years from the initial description of the syndrome milder features of the disease are recognized or extra-immunological clinical signs identified.

In the third group, NGS sequencing revealed multiple heterozygous genetic variants in each subject, some of them potentially involved in the immunological features. All the variants of this group are either single heterozygous or *in cis*, thus further studied are needed, including WES or microarray testing to try to find the second mutation or uncover mutation(s) in a separate gene that fully explain(s) the phenotype. In the patient 012, the PRF1 gene alteration was proven to have functional relevance,

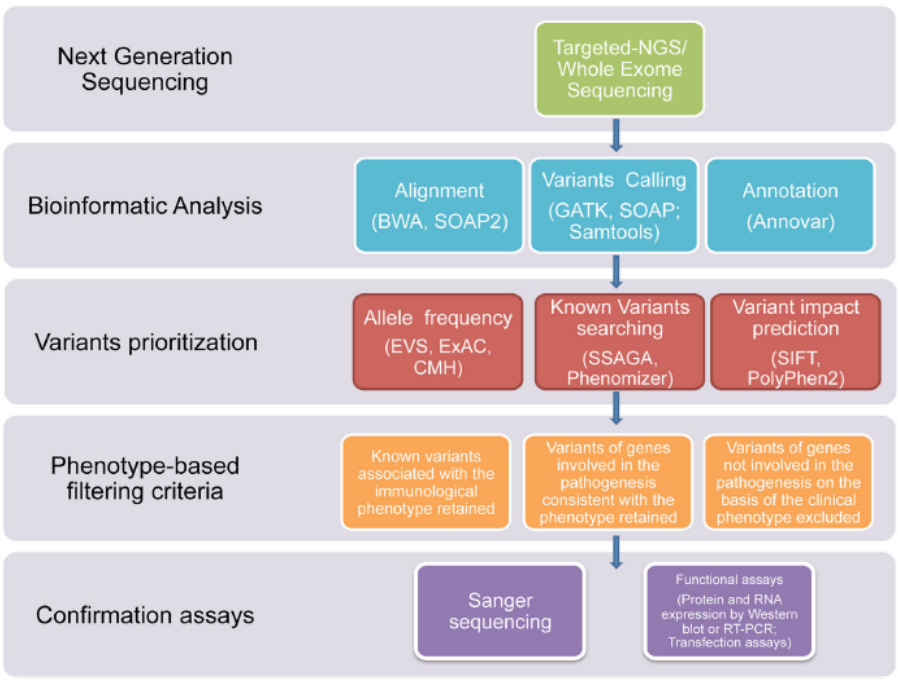


**FIGURE 1 | Flowchart of the filtering strategy**. A schematic overview of the approach/clear-cut strategy used to filter variants identified through T-NGS or WES in order to identify potentially causative mutations.

since perforin expression in NK cells was reduced. However, the patient was not placed in the group 1 since the two heterozygous PRF1 variations were in *cis* at the Sanger sequencing. Previously, our group has documented that this heterozygous variation may act as a susceptibility cofactor, which under certain circumstances may be associated with a functional alteration (42).

Finally, the fourth group included patients showing multiple genotypic alterations associated with complex phenotypes that could not fit into defined clinical phenotypes. In this group, no genotype–phenotype relationship was possible. However, since these cases are extremely rare, these data should likewise be collected in a database. The creation of such a database might improve the interpretation of NGS results in those cases currently interpreted as no causative of the disorder. The identification of different individuals with the same phenotype and mutations in the same array of genes would suggest that the sum of variations in different genes exerts a pathogenic role, either as causative or as modifier factor.

However, even though in our study in 7 out of the 45 patients, a diagnosis was achieved, it should be considered that in the majority of the patients, target NGS approach did not allow to identify the genetic basis of the disease. Several aspects should be considered to interpret this observation. The first limitation is the limited number of the genes included on our targeted NGS panel. Moreover, the sequencing techniques do not provide enough coverage for intronic, promoter, or regulator regions. To overcome these technical limitations, whole exome or genome sequencing might be better strategies to deeply investigate these cases as the second-line diagnostic tool (14). Moreover, all NGS techniques, due to the generation of short reads, show a low sensitivity to detect complex structural variations (deletions, insertions, and inversions), repeat sequences, or complex rearrangement (43).

Based on these considerations, the identification of genetic defects in patients with PIDs is still a major challenge, and the functional implication of the variation must be considered mandatory to definitely prove the relationship between the genetic alteration and the related phenotype.

Despite the above mentioned limitations, NGS technology represents a cost-effective and rapid first-line genetic approach for the evaluation of complex cases of PIDs. The advantage of this technique is the simultaneous sequencing of a panel of genes,

perhaps leading to the rapid identification of a diagnosis that may not have been otherwise considered using the traditional phenotype-driven approach. Overall, in spite of a moderately higher cost, at moment WES could represent the first-line approach to initial PID management. The sequential investigation of several candidate genes is, by comparison, a very time- and cost-consuming process. Prompt diagnosis shows an unquestioned clinical advantage, allowing initiation of appropriate and often life-saving treatment.

## AUTHOR CONTRIBUTIONS

VG, GG, EC, VL, RC, and AP identified the pts and performed the immunological and phenotypic characterization; IT, EF, and CS performed the NGS and WES exps; RDA, AP, and VL performed the functional exps and the molecular confirmation exps; RB and CP interpreted the results; VG and CP wrote the manuscript; and RB and CP directed the project.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00466

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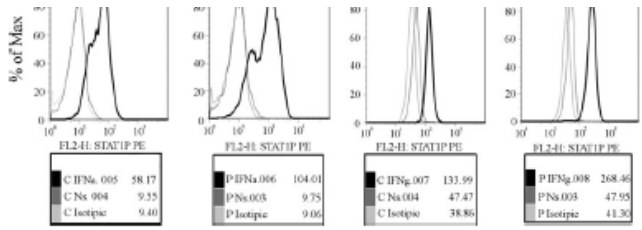
# Novel STAT1 gain-of-function mutation and suppurative infections

To the Editor,

Chronic mucocutaneous candidiasis (CMCC) is a heterogeneous group of disorders characterized by non-invasive persistent Candida species infections of the skin, nails, and mucous membranes. Heterozygous dominant gain-of-function (GOF) mutations in signal transducer and activator of transcription 1 (STAT1) have been described as causing impaired STAT1 dephosphorylation, diminished IL-17-producing T-cell numbers, and CMCC (1, 2). Here, we report on the case of a 17-year-old boy who presented to our Department for CMCC. He was born preterm (36 weeks) to healthy non-consanguineous parents from Italy, by a pregnancy complicated by threatened miscarriage and gestosis. Since childhood, he suffered from undocumented dermatologic alterations and, at 7 years of age, he was diagnosed as affected with mucocutaneous candidiasis. At 8 years of age, he suffered from a severe varicella infection, and since 11 years of age, the patient experienced recurrent herpetic infections of the genitals and limbs. Since the same period, he also suffered from recurrent suppurative eyelid infections (Fig. 1a) and cutaneous abscesses, unusual in this immunodeficiency, which developed on an otherwise healthy skin. The patient only experienced cutaneous abscess formation, while lymph nodes and inner organs were never involved. At 10 years of age, the patient presented with a prolonged (20 days) and severe gastroenteritis, which eventually led to severe dehydration. Familial history revealed no members with relevant fungal infectious diseases or immunodeficiencies. At the first evaluation, the patient showed oral thrush, onychomycosis (Fig. 1b), suppurative eyelid infection (Fig. 1a), furunculosis, and periodontitis. Cultures from the oral lesions, the nails, and the esophageal mucosa grew Candida albicans, sensitive to Azoles. Esophageal biopsy revealed the presence of fungal hyphae and chronic inflammatory infiltrate. Given the high susceptibility to Candida infection, a daily prophylactic treatment with fluconazole was started with a dramatic decrease in frequency and severity of fungal infections. Full-length sequencing of STAT1 genomic DNA identified a T387A STAT1 heterozygous mutation in the DNA-binding domain (DBD; Fig. 1c). This mutation has not been previously reported (3). None of the parents carried the mutation (Fig. 1d). To evaluate STAT1 phosphorylation, patient whole blood sample was stimulated with IFN-α (40,000 U/ml) or IFN-γ (1000 U/ml) and analyzed by flow cytometry. Both stimuli resulted in increased STAT1 phosphorylation in the patient $CD3^+$ T cells and $CD14^+$ monocytes, respectively, compared with control values (Fig. 1e). Routine laboratory evaluation revealed a normal or low–normal lymphocyte count and a normal T- and B-lymphocyte enumeration. The proliferative response to common mitogens (phytohaemagglutinin, PMA plus ionomycin, CD3 cross-linking) was normal. Total Ig and Ig subclasses levels and response to protein vaccines were normal. IgE levels were persistently elevated (684 kU/l). The study of the B-cell compartment revealed a number of $CD19^+$ cells within the normal range. The patient showed a normal representation of transitional ($CD3^-$ $CD19^+$ $CD24^+$ CD38hi$CD27^-$; 8.2%),



The proband is indicated with an arrow. (e) Patient whole blood sample stimulated with INF-α (40,000 U/ml) or IFN-γ (1000 U/ml) and analyzed by flow cytometry. Both stimuli result in increased STAT1 phosphorylation in the patient $CD3^+$ T cells and $CD14^+$ monocytes, respectively, compared with control values.




mature ($CD3^-$ $CD19^+$ $CD24^-$ CD38dim/lo$CD27^-$; 79.8%), and memory ($CD3^-$ $CD19^+$ $CD24^+$ $IgM^+$ $CD27^+$; 12%) B-cell subsets. However, memory B cells mostly included IgM and only a few cells were switched memory B cells (88% and 12% of the memory B cells, respectively). The function of B cells was studied *in vitro* by evaluating the response to the Toll-like receptor 9 ligand CpG. B cells from the patient carrying the *STAT1* mutation adequately proliferated in response to CpG, and $CD27^{bright}$ terminally differentiated plasma cells normally developed (Fig. S1). Accordingly, adequate levels of IgG and IgM were detected in the supernatants, even though only small amounts of IgA were secreted in the patient,

increased percentage of $CD4^+$ IFN-$\gamma^+$ cells (34.15% vs. 20.70%; Fig. 2a,b). We also studied TH17 *in vitro* differentiation and found a reduced (2.97% vs. 6.59%), but not abolished TH17 development in the patient (Fig. 2c). Finally, we studied the transcription levels of some STAT1 target genes (CXCL9, CXCL10, CCL5, and ICAM-1). The levels of CXCL9, CXCL10, CCL5, and ICAM-1 were higher than in the control either in unstimulated PBMC or following IFN-γ stimulation (Fig. 2e). The patient also had increased surface expression on unstimulated monocytes of MHC class II, whose transcription is under STAT1 control (Fig. 2d).

In this study, we reported on a patient with CMCC,

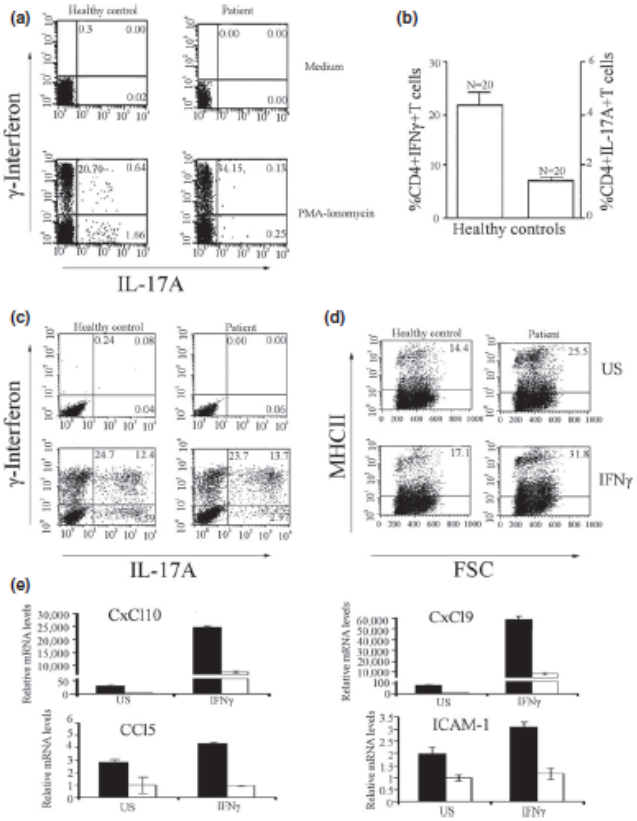



**Figure 2** *STAT1* GOF mutation impairs TH17 development and increases the expression of STAT1-regulated genes. (a) Percentage of $CD4^+$ $IL\text{-}17A^+$ and $CD4^+$ $IFN\text{-}\gamma^+$ cells following PMA plus ionomycin stimulation for 6 h. The patient shows a lower number of $CD4^+$ $IL\text{-}17A^+$ cells than the control (0.25% vs. 1.66%) and increased percentage of $CD4^+$ $IFN\text{-}\gamma^+$ cells (34.15% vs. 20.70%). (b) Percentage of $CD4^+$ $IFN\text{-}\gamma^+$ and $CD4^+$ $IL\text{-}17A^+$ cells in 20 healthy controls (values expressed as mean ± SD). (c) $CD4^+$ $IL\text{-}17A^+$ and $CD4^+$ $IFN\text{-}\gamma^+$ cell development after stimulation of $CD4^+$ cells, separated by positive selection using human CD4 microbeads, with anti-CD28, anti-CD3 X-L, IL-6, IL-1β, TGF-β1, IL-23 for 6 days in the patient and a healthy control. After 6 days, cells were split and cultured for further 6 days with the addition of IL-2. The patient shows a reduced (2.97% vs. 6.59%), but not abolished $CD4^+$ $IL\text{-}17A^+$ development. $CD4^+$ $IFN\text{-}\gamma^+$ development is comparable in the patient and control (23.7% vs. 24.7%). (d) MHC class II surface expression on unstimulated monocytes or after stimulation with IFN-γ. The patient shows increased MHC class II surface expression on either resting cells and after stimulation with IFN-γ as compared to the healthy control. (e) Real-time PCR analysis of the mRNA extracted from the patient PBMCs showing higher levels of CXCL9, CXCL10, CCL5, and ICAM-1 than in the control either in unstimulated PBMC or following IFN-γ stimulation.

to low–normal lymphocyte cell counts, and reduced levels of switched memory B cells (5).

STAT1 GOF mutations are considered responsible for very complex and variable phenotypes, characterized by susceptibility to herpetic (6) and fungal infections (7), autoimmunity, enteropathy, cardiac and vascular alterations, bronchiectasis (8), parodontitis, and failure to thrive (5, 9). In our patient, the clinical phenotype is dominated by recurrent furunculosis, parodontitis, and suppurative eyelid infections, mostly caused by *Staphylococcus* infections (10). As the hallmark in the infectious history of GOF mutations of STAT1 is considered the *Candida* infection, the case herein described further extends the complexity of the phenotype observed in these patients. In this patient, we also found an

increased transcription of pro-inflammatory molecules, as CXCL9, CXCL10, CCL5, and ICAM-1, which could help explain the pathogenesis of some features of this complex phenotype.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:
**Figure S1.** PBMCs cultured with medium or CpG were stained with antibodies to CD27 and IgM at day 7.

# Daily subcutaneous administration of human C1 inhibitor in a child with hereditary angioedema type 1

To the Editor,

Hereditary angioedema (HAE) is a rare autosomal-dominant inherited disorder, caused by local elevations of bradykinin due to a quantitative or qualitative deficiency of C1-INH resulting in recurrent mucosal or subcutaneous swelling attacks. Hereditary angioedema attacks can occur in all locations of the body and are potentially life-threatening if the face or larynx is affected. The diagnosis of HAE is based on clinical symptoms (e.g., severe abdominal pain or recurrent non-pruritic swelling of the skin or submucosal tissues lasting for 2–7 days) and laboratory screening with C4 (usually decreased in patients with HAE), C1-INH antigenic protein (decreased in HAE type 1) and C1-INH function (decreased in patients with HAE types 1 and 2). The majority of the patients benefit from an on-demand therapy (for review, see Ref. (1)). However, depending on the severity of disease, frequency of attacks, patient's quality of life, availability of resources, and failure to achieve adequate control by appropriate on-demand therapy, prophylactic treatment should be considered. Long-term prophylaxis with plasma-derived (pd)C1-INH concentrate requires frequent i.v. injections, in most cases twice per week (2, 3). S.c. infusions of pdC1-INH concentrate are thought to reduce this burden. First pre-clinical studies in adult patients with HAE reported on the safety and feasibility of s.c. administration of pdC1-INH concentrate with a bioavailability of functional C1-INH of 39.7% compared to i.v. administration (4). Recently,

increased transcription of pro-inflammatory molecules, as CXCL9, CXCL10, CCL5, and ICAM-1, which could help explain the pathogenesis of some features of this complex phenotype.

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# CHAPTER VII

## “Immunodeficiencies and Autoimmunity”

Even though Immunodeficiencies and autoimmunity may be considered two opposite conditions, deriving from different alterations of the immune system, several evidences suggested that PIDs are often associated with different autoimmune manifestations (95).

Autoimmunity in PIDs may be caused by different mechanisms, including defects of tolerance to self-antigens and persistent stimulation as a result of the inability to eradicate antigens.

This general immune dysregulation leads to compensatory and exaggerated chronic inflammatory responses that lead to tissue damage and autoimmunity.

Each PID may be characterized by distinct, peculiar autoimmune manifestations (96).

In the review published on *Frontiers in Pediatrics,* the main autoimmune manifestations and the pathogenetic mechanism underlying autoimmunity in a specific PID has are summarized.

A case report describing a skin vasculitis in a patient with APECED has been published on *BMC Pediatrics.*

frontiers in Pediatrics

MINI REVIEW
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# Unbalanced Immune System: Immunodeficiencies and Autoimmunity

*Giuliana Giardino, Vera Gallo, Rosaria Prencipe, Giovanni Gaudino, Roberta Romano, Marco De Cataldis, Paola Lorello, Loredana Palamaro, Chiara Di Giacomo, Donatella Capalbo, Emilia Cirillo, Roberta D'Assante and Claudio Pignata**

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Increased risk of developing autoimmune manifestations has been identified in different primary immunodeficiencies (PIDs). In such conditions, autoimmunity and immune deficiency represent intertwined phenomena that reflect inadequate immune function. Autoimmunity in PIDs may be caused by different mechanisms, including defects of tolerance to self-antigens and persistent stimulation as a result of the inability to eradicate antigens. This general immune dysregulation leads to compensatory and exaggerated chronic inflammatory responses that lead to tissue damage and autoimmunity. Each PID may be characterized by distinct, peculiar autoimmune manifestations. Moreover, different pathogenetic mechanisms may underlie autoimmunity in PID. In this review, the main autoimmune manifestations observed in different PID, including humoral immunodeficiencies, combined immunodeficiencies, and syndromes with immunodeficiencies, are summarized. When possible, the pathogenetic mechanism underlying autoimmunity in a specific PID has been explained.

**Keywords: autoimmunity, immunodeficiency, autoimmune hemolytic anemia, immune thrombocytopenia, systemic lupus erytematous**

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## INTRODUCTION

Immunodeficiencies and autoimmunity may be considered two opposite conditions, deriving from different alterations of the immune system. However, the evidence that primary immunodeficiencies (PIDs) are often associated with different autoimmune manifestations suggests that they could share common pathogenetic mechanisms, which result in a broad immune dysregulation.

Immune system becomes self-tolerant through two main mechanisms called central and peripheral tolerance. As for T cells, central tolerance takes place within the thymus and is mediated by medullary thymic epithelial cells (mTEC), which express tissue-specific antigens under the control of the the transcription factor autoimmune regulator (AIRE) (1–3). Developing T-cells recognizing self-antigens receive a signal to die via programed cell death and, thereby, are deleted, through negative selection, from the T-cell repertoire (4, 5). As for B cells, negative selection of autoreactive cells takes place within the bone-marrow. Different mechanisms, including immunological ignorance, anergy, and suppression through regulatory T cells (Treg) are implicated in the control of self-reactive cells, which escape central tolerance and reach the periphery. For example, the ligation of T-cell receptor (TCR), in the absence of costimulatory molecules, makes the cells inable to express effector functions like cytokine secretion, leading to anergy (6). The control of the expression of the costimulatory molecules CD80 and CD86 is a major mechanism of peripheral tolerance (6).

In some cases, the inability to eradicate foreign antigens may lead to an exaggerated chronic inflammatory responses and autoimmunity (7–10), through several mechanisms, including

molecular mimicry, by-stander activation, epitope spreading, and cryptic antigens.

Each PID is characterized by distinct, peculiar autoimmune manifestations (**Tables 1** and **2**), but the mechanisms may differ.

In this review, we will describe the main autoimmune manifestations observed in different PIDs, including humoral immunodeficiencies, combined immunodeficiencies, and syndromes with immunodeficiencies, and, when possible, we will try to explain the pathogenetic mechanism underlying autoimmunity in a specific PID.

## AUTOIMMUNITY IN HUMORAL IMMUNODEFICIENCIES

### Selective IgA Deficiency

Selective IgA deficiency (SIgAD) is the most common PID in humans (11). According to European Society for Immunodeficiencies (ESID) criteria, SIgAD is defined by the presence of serum IgA levels <0.07 g/l in the absence of IgG and IgM deficiencies, after the age of 4 years (12). Patients with SIgAD have an increased risk to develop allergies and autoimmune manifestations, including juvenile idiopathic arthritis, rheumatoid arthritis, thrombocytopenic purpura, hemolytic anemia, inflammatory bowel disease (IBD), Sjogren's disease, polyarteritis nodosa, systemic lupus erythematosus (SLE), celiac disease, and insulin-dependent diabetes mellitus (T1D) (**Table 1**) (13). Little is known about the pathogenesis of SIgAD and the predisposition to autoimmunity in these patients. Specific human leukocyte antigen (HLA) haplotypes, including 8.1, DR7, DQ2, DR1, and DQ5 have been identified in patients with SIgAD at higher risk autoimmune diseases (14), such as SLE, autoimmune thyroiditis, and celiac disease. In a recent study, the identification of single nucleotide polymorphisms in the IF1H1 gene encoding for an interferon inducible RNA helicase 1 protein and of a mutation in the CLEC16A gene in SIgAD patients has been associated with the development of autoimmune manifestations (14). Jacob et al. hypothesized that IgA exerts a protective role against autoimmunity. In particular, the interaction between the Fc fragment of IgA receptor and the immunoreceptor tyrosine-based activation motif deactivates the

**TABLE 1 | Autoimmune manifestations in humoral immunodeficiencies.**

1. SIgAD
   - Juvenile idiopathic arthritis
   - Rheumatoid arthritis
   - ITP, AHA
   - IBD
   - Sjogren's disease
   - Polyarteritis nodosa
   - SLE
   - Celiac disease
   - T1D
2. CVID
   - ITP, AHA
   - SLE
   - IBD
3. PRKCD deficiency
   - Glomerulonephritis
   - Polychondritis
   - Antiphospholipid syndrome
   - LES
4. LRBA deficiency
   - IBD
   - AHA, ITP
   - Granulomatous-lymphocytic interstitial lung disease
   - T1D
   - Neutropenia
   - Chronic autoimmune hepatitis
   - Eczema
   - Uveitis
   - Alopecia
5. Hyper-IgM syndrome
   - IBD
   - Seronegative arthritis
   - Hypothyroidism
   - SLE
   - Autoimmune hepatitis
   - ITP, AHA
   - T1D
   - Uveitis

*SIgAD, selective IgA deficiency; ITP, immune thrombocytopenia, AHA, autoimmune hemolytic anemia; IBD, inflammatory bowel disease; SLE, systemic lupus eritematous; T1D, type 1 diabetes mellitus; CVID, common variable immunodeficiency.*

**TABLE 2 | Autoimmune manifestations in combined immunodeficiencies and in syndromes with immunodeficiency.**

1. RAG-1/2 deficiency RMRP, ADA, IL2RG, Artemis, DNA ligase IV, ZAP70, and IL7Ra deficiency
   - Omenn syndrome (erythrodermia, alopecia, hepatosplenomegaly, and lymphoadenopathy)
2. PNP-deficiency, and mutations of ADA, DNA ligase IV, Cernunnos, ORAI1 and STIM1, and hypomorphic RAG1 mutations
   - AHA, ITP
3. Wiskott–Aldrich
   - AHA
   - Autoimmune neutropenia
   - Vasculitis
   - IgA nephropathy
   - Polyarthritis
   - IBD
4. DiGeorge syndrome
   - ITP, AHA
   - Autoimmune arthritis
   - Autoimmune hepatitis
   - Vitiligo
   - IDB
   - Autoimmune endocrinopathy
5. Ataxia telangiectasia
   - Psoriasis
   - Autoimmune thyroid disease
6. STAT1 gain of function
   - Autoimmune thyroid disease
   - IPEX-like phenotype (eczema, enteropathy, T1D, hypothyroidism, and growth hormone insufficiency)
7. STAT3 gain of function
   - Early onset autoimmunity (neonatal diabetes, enteropathy, desquamative interstitial pneumonitis, and posterior uveitis)

*ITP, immune thrombocytopenia; AHA, autoimmune hemolytic anemia; IBD, inflammatory bowel disease.*

pathways of immune response carrying this motif through a partial phosphorylation (15). Moreover, the evidence of antibodies to bovine milk proteins in over 60% of IgA deficient patients may help explaining the association between SIgAD and inflammatory diseases of gastrointestinal tract (16, 17).

## Common Variable Immunodeficiency

Common Variable immunodeficiency (CVID) is a heterogeneous group of disorders characterized by a primary antibody deficiency, usually manifesting between the second and fourth decades of life with a mean age at onset of 26.3 years (18). It is the second most common immunodeficiency with an estimated prevalence ranging from 0.073 to 0.977 living patients per 100,000 inhabitants (19). According to the ESID diagnostic criteria, CVID should be taken into account in presence of a marked decrease of IgG and IgA with or without low IgM levels (measured at least twice; <2SD of the normal levels for their age) (http://esid.org/Working-Parties/Registry/Diagnosis-criteria). Moreover, all of the following criteria should be fullfilled: poor antibody response to vaccines (and/or absent isohemagglutinins) or low switched memory B cells (<70% of age-related normal value); secondary causes of hypogammaglobulinaemia have been excluded diagnosis is established after the 4th year of life; no evidence of profound T-cell deficiency (http://esid.org/Working-Parties/Registry/Diagnosis-criteria). More than 25% of CVID patients develop autoimmune complications (18, 20). Other medical conditions may include gastrointestinal infectious or inflammatory disease, lymphadenopathy, splenomegaly, and hematological malignancies (21). Cytopenia is the most common manifestation. Immune thrombocytopenia (ITP) has been found in up to 14% of patients and autoimmune hemolytic anemia (AHA) in up to 7% (22). In most cases (about 60%), the cytopenia precedes the identification of hypogammaglobulinemia (23). SLE has been reported in some rare CVID patient (24), predominantly females (89%). In about 50% of patients, CVID developed within 5 years of the diagnosis of SLE (24). Some patients experience an improvement in SLE symptoms when hypogammaglobulinemia appears (24). Hypogammaglobulinemia can develop because of the use of immunosuppressive treatment (i.e., corticosteroids or immunosuppressants). Unlike CVID, the cessation of therapy should solve hypogammaglobulinaemia. Nevertheless, in some occasion, the duration of post-cessation hypogammaglobulinaemia can be very prolonged, making difficult to understand its origin (25). IBD has been reported in 6–10% of CVID patients (**Table 1**) (22). Many different alterations could help explain the predisposition to autoimmune manifestations. In a subgroup of CVID patients, IL-7 levels were found to be increased (26, 27). IL-7 plays a key role in the expansion of autoreactive T-cell clones in the lymphopenic host (26, 27). Moreover, reduced levels of switched memory B cells and increased levels of activated CD21-low B cells have been associated with autoimmune manifestations in CVID. Increased levels of CD21-low B cells have been identified in SLE, rheumatoid arthritis, and cryoglobulinemia, suggesting a role for these cells in the pathogenesis of autoimmunity (28–30). Most CVID patients present with elevated BAFF levels (27). Of note, increased BAFF levels sustain the expansion of CD21-low B cells in CVID (31). Moreover, studies show that overexpression of BAFF in mice leads to B-cell hyperplasia, splenomegaly, and autoimmunity (32, 33). Different genetic mutations, including TACI, ICOS, BAFF-R, CD20, and CD21 have been associated with increased risk of developing CVID (34–40). Among these genetic alterations, autoimmunity is most common in TACI alterations [18/50 (36%) vs. 112/490 (23%) in wt TACI CVID], in particular, heterozygous C104R mutations (11/20 patients, 55%) (41).

## PRKCD Deficiency

A CVID-like disorder associated with multiple features of immune dysregulation, including glomerulonephritis, lymphadenopathy, relapsing polychondritis, and antiphospholipid syndrome has been recently described in a 12-year-old patient born to consanguineous parents of Turkish origin (42). Genetic studies revealed a mutation of *PRKCD* gene, leading to a complete absence of the protein. PRKCD deficiency has also been reported in three siblings with LES (**Table 1**) (43). PRKCD plays a key role in the regulation of cell survival, proliferation, and apoptosis (44). PRKCD deficiency in mice seems to be related to a defective deletion of autoreactive B cells during B-cell development, due to impaired proapoptotic extracellular signal-regulated kinase signaling (45, 46).

## LRBA Deficiency

LPS-responsive beige-like anchor protein (LRBA) deficiency is a novel PID caused by either homozygous or compound heterozygous mutations in *LRBA* that abolish LRBA protein expression. This PID is characterized by early onset hypogammaglobulinemia, autoimmune manifestations, susceptibility to IBD, and recurrent infections (47). However, it has been also described in patients with IBD with or without antibody deficiency (48, 49), in patients with autoimmune manifestations without hypogammaglobulinemia (50), or in patients with immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX)-like disorder (51). The main clinical manifestations of LRBA deficiency are immune dysregulation (95%), followed by organomegaly (86%) and recurrent infections (71%). The most common autoimmune manifestations are enteropathy (59%), AHA (50%), and ITP (50%). A lower number of patients presented granulomatous-lymphocytic interstitial lung disease (36%), T1D or neutropenia (22%), chronic autoimmune hepatitis (13%), eczema and uveitis (9%), and alopecia (4.5%) (**Table 1**). LRBA is a highly conserved multidomain protein implicated in regulating endosomal trafficking, cell proliferation, and survival. LRBA deficiency is associated with increased apoptosis and altered phenotype of Treg cells, which express lower levels of key effector proteins involved in Treg cell suppression, such as CD25 and CTLA-4. This results in decreased frequency, aberrant phenotype, and decreased suppressive function of such cells. These alterations might play a critical role in the ubiquitous autoimmune manifestations of the disease.

## CTLA-4 Haploinsufficiency

CTLA-4 haploinsufficiency has been recently associated with lymphoproliferation, lymphocytic infiltration, autoimmunity,

peripheral B-cell lymphopenia, hypogammaglobulinemia, and increased CD21lo B cells (52). In mouse models, homozygous CTLA-4 deficiency leads to a lethal autoimmune phenotype characterized by multiorgan lymphocytic infiltration and destruction (53, 54) resambling FOXP3 deficiency (55–57). CTLA-4 plays a key role in immune tolerance. Recent studies show that CTLA-4 is able to suppress the expression of CD80 and CD86 from antigen presenting cells (APCs) via transendocytosis (58). The depletion of the costimulatory ligands reduces T cell activation (59).

### Activated Phosphoinositide 3-Kinase δ Syndrome

Activated phosphoinositide 3-kinase δ syndrome (APDS) 1 and 2 are PID resulting from autosomal dominant mutations in PI3KCD and PIK3R1, respectively (60, 61). Autoimmune manifestations are reported in 34% of APDS1 patients. The clinical manifestations included cytopenias (AHA or tri-lineage cytopenia), glomerulonephritis, exocrine pancreatic insufficiency, autoimmune thyroid disease, seronegative arthritis, recurrent pericarditis, sclerosing cholangitis, and gastrointestinal nodular mucosal lymphoid hyperplasia (61). Autoimmune manifestations have been reported in the 17% of the APDS2 patients. They included ITP, AHA, Evans syndrome, T1D, chronic arthritis, autoimmune hepatitis, and chronic eczema (60). PI3Kδ is implicated in the regulation of Treg cell function. Studies suggest that PI3K is an important target for the treatment of different autoimmune conditions.

### Hyper-IgM Syndrome

Hyper-IgM syndrome (HIGM) is a group of disorders characterized by alterations of immunoglobulin receptor isotype switching, leading to normal or elevated IgM antibody and very low IgA, IgG, and IgE antibodies (62). Alterations in different genes implicated in CD40-CD40L pathway involved in B cell activation, class switch recombination or somatic hypermutation have been identified in HIGM. Seven different forms of HIGM have been till now described. Most of the cases (65–70%) are due to mutations of the gene encoding for CD40 ligand (CD40L) on the X chromosome, leading to HIGM1 (63). The other forms are due to mutations of AID (HIGM2), CD40 (HIGM3), UNG (HIGM5), NEMO (HIGM6), and IkBα (HIGM7). No genetic defect has been so far identified for HIGM4.

Autoimmunity has been described in all forms of HIGM. HIGM1 patients have an increased risk to develop IBD, seronegative arthritis, hypothyroidism, and SLE (64). In the 21% of patients affected with HIGM2 autoimmune hepatitis, ITP, T1D, IDB, and uveitis have been described (65). In addition, in patients with NEMO defects, AHA, IBD, and arthritis have been described (**Table 1**) (66). Studies on transgenic mouse models suggest that CD40–CD40L interactions is involved in the elimination of autoreactive B cells (67). In fact, an increase of circulating polyreactive B cells and a significant decrease of CD25+Foxp3+Treg cells have been reported in CD40L-deficient patients suggesting defects of the peripheral B-cell tolerance mechanism. An imbalanced production of cytokines, including as IL-1, IL-8, IL-6, IL-10, IL-12, and tumor necrosis factor (TNF)-α may be observed in CD40-deficient patients (68). This impairment is the consequence of the involvement of CD40–CD40L interaction in T-cell dependent macrophage-mediated immune response, implicated in the maturation of dendritic cells and regulation of the T-cell activation. The transcription factor NFkB plays a key role in the regulation of pro-inflammatory responses. Recent studies suggest that gut epithelial cells are directly implicated in the control of epithelial integrity and the regulation of the interaction between the mucosal immune system and gut microflora. In mice, NEMO deficiency causes a severe chronic intestinal inflammation, which has been associated with apoptosis of colonic epithelial cells, impaired expression of antimicrobial peptides, and translocation of bacteria into the mucosa. The chronic inflammatory response observed within the colon, is dominated by innate immune cells, as suggested by the upregulation of IL1b, IL6, TNF, and Ccl2 and by the infiltration of large numbers of dendritic cells and granulocytes in the colon. Eventually, also T lymphocytes are involved, as suggested by the presence of lymphoid follicles and a massive infiltration with CD4+ T cells in the gut mucosa.

## COMBINED IMMUNODEFICIECIES

Severe combined immunodeficiency (SCID) is a group of different PIDs characterized by a severe deficiency of the cellular and humoral immune system. SCID phenotype may be due to a variety of different mutations. From a clinical point of view, SCID is characterized by recurrent severe infections, chronic diarrhea, and failure to thrive (69, 70). The clinical presentation may drive the diagnosis toward a specific molecular cause of SCID (69). Patients affected with SCID often develop autoimmune manifestations. This may appear surprising in that SCID patients, who are unable to mount any immune response to foreign pathogens, may paradoxically develop autoimmune phenomena. Alterations in both central and peripheral tolerance have been described in SCID patients (71).

### Autoimmunity in Omenn Syndrome

Omenn syndrome (OS) is a SCID inherited in an autosomal recessive manner, caused by homozygous or compound heterozygous mutations in recombinase activating gene 1 (RAG1) or RAG2, implicated in V(D)J recombination, which represents a crucial step in T- and B-cell differentiation. OS has also been associated with hypomorphic mutations in other different genes, including RMRP, ADA, IL2RG, Artemis, DNA ligase IV, ZAP70, and IL-7Ra deficiency (72, 73). Signs of OS, including oligoclonal T-cell expansion, generalized rash, and lymphadenopathy have been reported in some patient affected with DiGeorge syndrome. This rare condition is known as atypical complete DiGeorge syndrome (74). Apart from recurrent infections, patients affected with OS also show features of autoimmunity, including erythrodermia, alopecia, hepatosplenomegaly, and lymphadenopathy (**Table 2**). The hallmark of the syndrome is the expansion and activation of a peripheral oligoclonal population of autoreactive T cells, due to defective central (75, 76) and peripheral tolerance mechanisms (77). Studies suggest

that in OS, defective AIRE expression may lead to inadequate expression of tissue-specific self-antigens by mTEC, impairing central tolerance. In these patients, the T-cell compartment is composed by a high proportion of autoreactive T cells, which are able to expand in peripheral tissues leading to the clinical symptoms. Similarly, alterations in central tolerance may be implicated in the pathogenesis of immune manifestations also in PIDs characterized by ineffective thymopoiesis, such as the DiGeorge syndrome or in SCID characterized by partial defects of the T-cell maturation, such as IL-7alfa, common γ chain, or ARTEMIS defects. The persistent infectious/inflammatory state and the presence of immunologic "space," which increases the ability of T cells to respond to an excess of cytokines or antigens, impairs peripheral tolerance in SCID patients. Treg population may be also affected in SCID patients.

### Autoimmune Manifestations in SCID Due to IL7R Mutations

IL7Ra deficiency is responsible of the majority of T-B+NK+ cases (72) characterized by an increased susceptibility to severe and opportunistic infections. In a few cases, autoimmune manifestations have been reported (72, 73). Autoimmune manifestations presented with OS in one infant (73), and cytopenias in three other cases. Autoimmune cytopenias have been also described in some patients with PNP-deficiency, and mutations of ADA, DNA ligase IV, Cernunnos, and hypomorphic RAG1 mutations (**Table 2**) (69, 78–82).

### Ca++ Channellopathies Due to Mutations in ORAI1 and STIM1

Null or loss-of-function mutations in ORAI1 or STIM1 are associated with a SCID-like disease characterized by recurrent and chronic infections, autoimmunity, ectodermal dysplasia, and muscular hypotonia in the presence of numerically intact T, B, and NK cells. Symptoms usually manifest in the first year of life. Lymphoproliferation, AHA, and ITP are very common in patients with STIM1 mutations (**Table 2**). Autoimmunity may derive from alterations of negative selection of autoreactive T cells and/or B cells during their development. In fact, Ca2+ signals are implicated in TCR and BCR signaling and thus potentially influence the selection thresholds in immature T and B cells. Moreover, a reduced frequency of Treg cells has been observed in STIM1-deficient patients (83, 84) and in one patient with ORAI1 p.R91W mutation.

## SYNDROMES WITH IMMUNODEFICIENCY

### Wiskott–Aldrich

Wiskott–Aldrich syndrome is a very rare immunodeficiency, characterized by thrombocytopenia, eczema, and recurrent bacterial infections appearing in the first months of life. Other features includes humoral and cellular immunodeficiency, defects of the innate immunity (85–87), increased risk to develop autoimmune manifestation and malignancies, impaired apoptosis (88, 89), and defective cell motility (90). The gene responsible for WAS (WASP) is located on the X chromosome and encode for WASP protein, which is only expressed in the cytoplasm of hematopoietic cells. WASP protein plays a major role in the transduction of the signals from the cell surface to the actin cytoskeleton, which regulates actin polymerizationand the formation of actin filament (91, 92). WAS patients are at a higher risk of developing autoimmunity and most of WAS patients (about 40%) are affected by at least one autoimmune manifestation (93, 94). The most common autoimmune manifestations include AHA, autoimmune neutropenia, vasculitis, and IgA nephropathy with or without the association with Henoch–Schönlein purpura, polyarthritis and IBD (**Table 2**) (87, 93–95). Studies suggest that a defect in Treg cells could be implicated in the pathogenesis of autoimmune manifestations (96, 97). In fact, Treg cells, isolated from WAS patients, show a reduced ability to suppress effector T-cell proliferation and IFN-γ production (98, 99). On the contrary, Treg cell development is not impaired in these patients. In addition, Treg cells from WASp−/− mouse show a reduced granzyme B secretion, which results in the inability to suppress B-cell proliferation and apoptosis. Furthermore, studies on mouse models show that Treg cells from WASp−/−mouse are not able to prevent the development of autoimmunity in scurfy mice (Foxp3-deficient) (98–100). Also B cells may be implicated in the pathogenesis of autoimmune manifestations in WAS patients. Studies show that selective deletion of WASP in B cells leads to the production of autoantibodies and the development of autoimmunity (101, 102).

### DiGeorge Syndrome

Autoimmune manifestations have been reported in about the 10% of patients with DiGeorge syndrome (103–105). Autoimmune disorders include mainly autoimmune cytopenias (ITP, AHA) (106–108), autoimmune arthritis (107), autoimmune hepatitis, vitiligo, IDB, and autoimmune endocrinopathy (**Table 2**) (109). Impaired T-cell development in an abnormal thymus may result in altered central tolerance and escape of self-reactive T. Thymic abnormality may also result in impaired generation of Treg (96, 110, 111).

### Ataxia Telangiectasia

Patients with ataxia telangiectasia (A-T) have increased frequency of autoimmune disorders (112), including psoriasis and autoimmune thyroid disease (**Table 2**). Loss of suppressor T-cell function has been described as responsible for the development of autoimmune disease.

### STAT1 Gain of Function

Increased incidence of autoimmunity has been reported in heterozygous STAT1 gain-of-function (GOF) mutations (113). The main clinical features of the syndrome include chronic mucocutaneous candidiasis (CMC) (114–118), disseminated coccidioidomycosis, and histoplasmosis (116, 119), recurrent sinopulmonary infections and pneumonias (with or without bronchiectasis), herpes virus infections, blood-borne infections, squamous cell cancer, and cerebral aneurysms (116, 120). The most common autoimmune manifestation is thyroiditis, but in

some case patient may show an IPEX-like phenotype (121). Number and function of Treg cells are usually normal and the pathogenesis of IPEX-like disease remains unclear (121).

### STAT3 Gain of Function

Recent studies show that activating STAT3 mutations may lead to autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease (122). The autoimmune manifestations are early onset and include neonatal diabetes and some rare disorders, such as desquamative interstitial pneumonitis and posterior uveitis (123). Patients with activating STAT3 mutations show a reduced number of Th17 cells, decreased IL-17 production, and deficiency of Treg, NK, and dendritic cells (123). Autoimmunity may develop as a consequence of the impaired Treg development.

## CONCLUSION

Autoimmunity and immunodeficiencies represent two opposite conditions, which may coexist in the context of a general immune dysregulation. Even though different mechanisms have been identified to explain autoimmunity in PIDs, the pathogenesis of autoimmunity remains unexplained in most of the cases. Considering this strong association, underlying immunodifiency should be always excluded in particular in presence of early onset or multiple autoimmune manifestations (124).

## AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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CASE REPORT Open Access

# Cutaneous vasculitis in patients with autoimmune polyendocrine syndrome type 1: report of a case and brief review of the literature

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**Abstract**

**Background:** Autoimmune polyendocrine syndrome type 1, also known as autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy, is a rare autosomal recessive disease due to pathogenic variants in the *AIRE* gene. Classic features of the syndrome are mucocutaneous candidiasis, chronic idiopathic hypoparathyroidism and Addison disease. However, other endocrine and non-endocrine components, may occur with a different prevalence. In addition to ectodermal features, which are quite common features of the disease, APS 1 patients may experience other types of skin alterations, such as vasculitic skin rash. An early diagnosis of APS 1 can be very challenging, due to the high clinical heterogeneity, and a considerable delay may occur between the appearance of symptoms and the diagnosis.

**Case presentation:** We report on a girl affected by APS 1 who presented with cutaneous vasculitis when she was seven-months old, some years before the onset of the common components of the disease.

**Conclusion:** Clinical picture of APS 1 may be characterized by isolated rare or atypical autoimmune or immune-mediated manifestations, even years before the onset of the classic components of the disease. Among these uncommon features, skin rashes of variable form and duration may occur, most of them being associated with histopathological features of vasculitis. Our case suggests that cutaneous vasculitis may represent a first sign of APS 1. The clinical significance of cutaneous vasculitis in the context of APS 1 is still debated. It may represent a rare, unusual, early component of the disease or a clinical manifestation secondarily related to the typical APS 1 components (i.e. autoimmune thyroid disease), which are frequently associated with rheumatologic-like signs and symptoms. Alternatively, it may be the expression of an independent disease co-occuring with APS 1. In conclusion, our case suggests that children presenting with unexplained vasculitic skin rash should be followed-up in order to early identify APS 1.

**Keywords:** APS 1, *AIRE*, Cutaneous vasculitis, Autoimmunity

## Background

Autoimmune polyendocrine syndrome type 1 (APS 1), also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), is a rare autosomal recessive disease caused by pathogenic variants in the autoimmune regulator (*AIRE*) gene. *AIRE* encodes for the homonymous protein, AIRE, which acts as a regulator of the process of gene transcription and is involved in the mechanisms of deletional central (and presumably peripheral) tolerance. AIRE deficiency leads to the escape and extra-thymic spreading of autoreactive T-cell clones: this creates the basis for the onset of the autoimmune attack against several tissue-specific self-antigens [1].

The clinical diagnosis of APS 1 is defined by the presence of at least two components of the classic triad, which is given by chronic mucocutaneous candidiasis (CMC), chronic idiopathic hypoparathyroidism (HPT) and Addison disease (AD). The disease generally begins in childhood

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and CMC is the first component appearing by five years of age, followed by HPT and then by AD. Other endocrine and non-endocrine components, such as hypergonadotropic hypogonadism, hypothyroidism, type 1 diabetes, gastrointestinal dysfunction, autoimmune hepatitis, asplenia and various ectodermal abnormalities (interstitial keratitis, alopecia, vitiligo, nail dystrophy and dental enamel hypoplasia), may occur with a different prevalence [2-5]. In addition to ectodermal features, which are quite common features of the disease, APS 1 patients may experience other types of skin alterations. Indeed, in a restricted number of cases a maculopapular, or morbilliform, or urticaria-like skin rash, eventually accompanied by fever, splenomegaly and arthralgia, has been reported [2,3,6-18]. When performed, biopsy of the above lesions has revealed perivascular, lymphoplasmacytic infiltrates in most of the cases [3,11,12,18]. Whether skin involvement represents the expression of a direct autoimmune attack, or an unrelated event still remains to be defined.

Here we report on a challenging diagnosis of APS 1 in a patient who presented at a very early age with a urticarial skin rash, with histopathological evidence of vasculitis at skin biopsy, some years before the onset of other classic components of the disease.

## Case presentation

A 7-month-old female of non-consanguineous parents, presented with a skin rash consisting of purple plaques (maximum diameter 4 cm) with irregular and erythematous margins, which were localized to the trunk and limbs. The child also had mild splenomegaly and relapsing episodes of joint pain with fever. Skin biopsy showed inflammatory infiltrates within and around the walls of small vessels with signs of endothelial damage in the form of endothelial swelling, thus confirming a diagnosis of vasculitis. The child underwent a diagnostic work-up, which showed increased levels of C-reactive protein (27 mg/dl; n.v. <0.5), erythrocyte sedimentation rate (66 mm/hour; n.v. <10) and immunoglobulins (IgG 30.9 g/l; n.v. 1.7-10.7 and IgM 1.63 g/l; n.v. 0.3-1.3). C3 and C4 complement factors were within the normal range (C3 1.13 g/l; n.v. 0.6-1.8 and C4 0.7 g/l; n.v. 0.07-0.7). Antibodies against common infectious agents were negative. The percentage of double negative T lymphocytes (CD3 + CD4-CD8-), the lymphocyte response to mitogens and lymphocyte sensitivity to FAS-induced apoptosis were all normal. Anti-nuclear (ANA), perinuclear (p-) and cytoplasmic (c-) anti-neutrophil cytoplasmic (ANCA), anti-thyroid, anti-double stranded (DS) DNA, anti-phospolipids antibodies were all undetectable. Skin lesions regressed spontaneously by the second year of life; biochemical abnormalities also normalized during the follow-up except levels of IgG and IgM which remained elevated. The patient was then referred at the age of 5 years because of recurrent oral candidiasis, alopecia of eyelashes and eyebrows, autoimmune thyroiditis, abdominal pain and diarrhea. Despite a mild increase in serum TSH levels (6 mIU/l), free-T4 (FT4) levels were normal, and the patient did not require levothyroxine treatment [19].

Based on the persistence of candidiasis, cutaneous manifestations and autoimmune thyroiditis, direct sequencing of the *AIRE* gene was performed, revealing 47C > T and 232 T > A variants in the exons 1 and 2, respectively, thus confirming the diagnosis of APS 1. These variants were inherited from the parents. Autoantibodies evaluation revealed positive anti-thyroid, adrenal cortex, 17- and 21-hydroxylase, gastric parietal cells, tryptophan hydroxylase, side-chain cleavage, L-amino acid decarboxylase, and steroid-producing cells antibodies (Table 1). Antibodies against the IA-2 tyrosine phosphatase-like protein, insulin, and glutamic acid decarboxylase were negative on several controls. Anti-interferon antibodies were first evaluated when she was 9 years-old, and were found to be positive.

During the follow-up the patient developed other signs and symptoms of the disease. At the age of 9 years, she was noted to have areas of vitiligo and signs of ectodermal dystrophy, such as dental enamel and nail dysplasia. HPT was diagnosed at the age of 9 years on the basis of low calcium (1.5 mmol/l) and parathyroid hormone levels (7 pg/ml, n.v. 10–65), and a treatment with calcitriol and calcium supplementation was started. At the age of 11 years, increased levels of ACTH (150 pg/ml; n.v. 10–130) and renin (184 pg/ml; n.v. 1.8-3.3 pg/ml), with reduced cortisol peak (108 ng/ml) after ACTH stimulation test, associated with presence of anti-adrenal cortex, 17- and 21-hydroxylase antibodies, led to the diagnosis of AD; therefore, glucocorticoid and mineralcorticoid replacement therapy were started. Six months later she also started levothyroxine treatment due to a further increase in TSH values (TSH 15 mU/ml), with reduced values of FT4 (0.6 ng/dl; n.v. 0.9-1.7). Patient's pubertal development was normal and she experienced menarche at the age of 10 years. However, at the age of 12 she had secondary amenorrhea with increased gonadotropin levels (FSH 50 mUI/ml; LH 35 mUI/ml). According to positivity of anti-steroid-producing cells antibodies, a diagnosis of premature ovarian failure due to autoimmune oophoritis was made, therefore she started hormonal replacement therapy with estrogens and progestins.

The clinical follow-up was also marked by an increase in the extent of the alopecia, which affected over time a large part of the scalp. Table 2 summarizes the clinical components occurring over time in our patient, according to the age of onset.

## Discussion

We report on a child with APS 1 who presented with cutaneous vasculitis at a very early age, more than one year before the onset of the typical features of the disease.

**Table 1 First detection of specific autoantibodies compared to the age of onset of APS 1 components**

| Specific autoantibodies | Age at first test (years) | Age at first detection (years) | Related APS 1 component | Onset of APS 1 component (years) |
|---|---|---|---|---|
| Anti-thyroglobulin | 5 | 5 | Autoimmune thyroiditis | 5 |
| Anti-thyreoperoxidase | 5 | 5 | Autoimmune thyroiditis | 5 |
| Anti-parietal cells | 7 | 7 | Autoimmune gastritis | - |
| Anti-adrenal cortex | 7 | 7 | Addison disease | 11 |
| Anti-21-hydroxylase | 8 | 8 | Addison disease | 11 |
| Anti-17alpha hydroxylase | 8 | 8 | Addison disease | 11 |
| | | | Ovarian failure | 12 |
| Anti-P450 side chain cleavage | 8 | 8 | Addison disease | 11 |
| | | | Ovarian failure | 12 |
| Anti-steroid-producing cells | 8 | 8 | Ovarian failure | 12 |
| Anti-tryptophan hydroxylase | 9 | 10 | Autoimmune hepatitis | - |
| | | | Autoimmune enteropathy | 5 |
| Anti-L-aminoacid decarboxylase | 9 | 10 | Vitiligo | 9 |
| | | | Autoimmune hepatitis | - |

Some clinical aspects and genetics of the patient described have been already included in a small case series described by Capalbo et al. [20] and within an Italian case series described by Mazza et al. [4]. However, these two papers focused on the genetics and the phenotypic heterogeneity of APS 1 patients originating from the same geographic area (the former) and from several Italian regions (the latter), and the clinical counterpart of our patient was only briefly and partially discussed.

Aim of the current paper was to describe in detail this patient with unusual presentation of APS 1, and to report information of its clinical course. Indeed, in our opinion such case raises interesting issues regarding the eventual involvement of the skin in APS 1 and aware physicians to consider the diagnosis of APS 1 when evaluating a patients with unexplained cutaneous vasculitis.

APS 1 is characterized by high phenotypic heterogeneity, with a great variability in the number of the clinical manifestations and the age at onset of the disease even between patients with the same genotype [3-5]. Indeed, the first clinical signs and/or symptoms may occur from the first months of life up to adulthood [21], the earlier presentation being generally associated with a more severe phenotype and a higher number of clinical components [1]. Although in its typical form APS 1 presents with at least one of the classic triad, some patients suffer from several minor manifestations of the disease for many years before one of the first major components occurs [2].

**Table 2 Clinical course of APS 1 in our patient**

| APS 1 component | Age of onset (years) |
|---|---|
| Cutaneous vasculitis | 0.7 |
| Mucocutaneous candidiasis | 2 |
| Abdominal pain with stipsis and diarrhea | 5 |
| Autoimmune thyroiditis | 5 |
| Alopecia | 5 |
| Ectodermal dystrophy | 9 |
| Vitiligo | 9 |
| Chronic idiopathic hypoparathyroidism | 9 |
| Addison disease | 11 |
| Ovarian failure | 12 |

When a rare or atypical component is the presenting feature of the disease, the diagnosis of APS 1 can be challenging and a considerable delay may occur between the appearance of symptoms and the diagnosis [22].

Although skin rash represents a rare manifestation of APS 1, to date a few patients with different forms of cutaneous eruptions have been described (Table 3) [2,3,6-18]. Only the minority of the cases reported were less than 1 year old [3,8,15].

Reports from Finnish population showed that 14% (13 out of 91 patients) of their cohort may exhibit periodic morbilliform, maculopapular or urticarial skin rashes with fever and/or arthralgia, appearing at age 0.7-31 years and lasting for 0.2-1.2 yr. Five out of these 13 patients, aged 0.7-1.2 years, showed high plasma IgG levels. Moreover, four patients underwent skin biopsy, revealing a lymphoplasmacytic vasculitis in two and no specific pathology in the other cases. The same author hypothesized an autoimmune pathogenesis of such component [3].

In another large series of 41 APS 1 subjects from Italy, only one patient was described as having cutaneous vasculitis [2].

**Table 3 Previous reports of skin involvement in APS 1 patients**

| Author and year of publication | N. of cases | Age at the onset of skin lesions (yrs) | Aspect of skin lesions | First typical component, age at onset (yrs) | Biopsy |
|---|---|---|---|---|---|
| **Case series** | | | | | |
| Craig JM et al. 1955 [7] | 1/3 | 2.8 | erythema marginatum/recurrent skin rash | CMC, 3 | increased melanin content |
| Betterle C et al. 1998 [2] | 1/41 | n.a. | n.a. | n.a. | n.a. |
| Perheentupa J 2006 [3] | 13/91 | 0.7–31 | fleeting maculopapular, morbilliform, or urticarial rash | n.a | 2/4 biopsies revealed vasculitis |
| Trebušak Podkrajšek K et al. 2008 [6] | 1/11 | 2 | n.a. | HPT, 7.5 | n.a. |
| Posovszky C et al. 2012 [15] | 2/13 | 1 | n.a. | CMC, 2.0 | n.a |
| | | 5 | chronic recurrent urticaria | CMC, 3.0 | |
| **Case reports** | | | | | |
| Quinto MG et al. 1964 [8] | 1 | 0.9 | multiform erythema | HPT + CMC, 4.0 | n.a. |
| Stickler GB et al. 1965 [9] | 1 | 7.6 | evanescent trunkal macular rash | HPT + CMC, 9.1 | n.a. |
| Spörkmann K-H et al. 1990 [10] | 1 | 1.2 | multiform erythema | HPT, 3.0 | n.a. |
| Garty B 1998 [11] | 1 | 22 | erythema annulare centrifugum | HPT, 5 | lymphohistiocytic vasculitis |
| Füchtenbusch M et al. 2003 [12] | 1 | 31 | purpuric subepidermal nodules progressing in deep cutaneous ulcers | HPT, 3.0 | panniculitis and lymphocytic vasculitis |
| Kapelari K et al. 2004 [13] | 1 | 16 | photosensitive facial rash (diagnosis of systemic lupus erythematosus) | n.a. | n.a. |
| Hoorweg-Nijman G et al. 2008 [14] | 1 | 1.2 | n.a. | HPT, 9.0 | n.a. |
| Montin D et al. 2008 [16] | 2 | 1.1 | urticarial rash (vasculitic rash) | CMC, 1.5 | n.a. |
| | | 7 | urticarial rash | CMC, 7.0 | |
| Rodríguez Sánchez De La Blanca A et al. 2012 [18] | 1 | 6 | photosensitive rash | HPT, 9.0 | n.a. |
| O' Gorman CS et al. 2013 [17] | 1 | 1 | intermittent urticarial rash | HPT + CMC, 1 | lymphocytic vasculitis |

CMC: chronic mucocutaneous candidiasias; HPT: chronic idiopathic hypoparathyroidism; n.a.: not available.

In a cohort of 11 APS 1 patients from different European countries (Serbia, Slovenia and Germany) the authors described a Serbian APS 1 girl first presenting with recurrent episodes of high fever, accompanied by cutaneous rash and arthralgias at the age of two years, who was diagnosed with systemic juvenile rheumatoid arthritis. This patient also suffered from asthma-like dyspnea and developed the first major APS 1 component (HPT) only when she was 7.5 years old [6].

Skin biopsy was performed in only a few cases, revealing in most, but not all, evidence of an underlying vasculitis [3,11,12,18]. For this reason, the prevalence of cutaneous vasculitis in the context of APS 1 is unknown and it might to be higher than that reported so far. As for our patient, vasculitic skin rashes in patients with APS 1 have been previously reported to be associated with other signs and/or symptoms, such as therapy-resistant and/or recurrent fever [6,7,9,10,13-16,18], polyarthritis [15], arthralgia [3,6,12], hepato-splenomegaly [7,16] and photosensitivity [13,17], as well as with laboratory and/or histological abnormalities such as hypergammaglobulinemia [9,13,16], elevated erythrocyte sedimentation rate [9,13], positive rheumatoid factor [15], traces of cryoglobulinemia [2] and panniculitis [12].

The clinical significance of cutaneous vasculitis in the context of APS 1 is still debated. It may represent a rare, unusual, early component of the disease or a clinical manifestation secondarily related to the typical APS 1 components (i.e. autoimmune thyroid disease), which are frequently associated with rheumatologic-like signs and symptoms. Alternatively, it may be the expression of an independent disease co-occuring with APS 1.

In this regard, taking into account that a complex clinical picture including skin rash may continue for years before some of the classic APS 1 components appear, it is not surprising that some patients have been initially suspected or diagnosed as having rheumatologic diseases like juvenile rheumatoid arthritis and Wissler-Fanconi syndrome [6,10] or other autoimmune disorders such as autoimmune hepatitis [15]. In our patient, the presence of early-onset cutaneous vasculitis, mild splenomegalia, and serum hypergammaglobulinemia first suggested a diagnosis of autoimmune lymphoproliferative syndrome (ALPS). ALPS is a chronic, non-malignant lymphoproliferative disorder due to mutations in the genes involved in apoptosis. As for APS 1, it presents in the first years of life (usually by 5 years of age) and its natural history is characterized by the development of multiple autoimmune manifestations [23]. However, in our case the normal lymphocyte sensitivity to FAS-induced apoptosis and the absence of double negative T cells (CD3 + CD4-CD8-), which represent the immunological hallmark of the disease, definitely ruled out a diagnosis of ALPS. The diagnosis of APS 1 was suspected only when recurrent oral candidiasis and several autoimmune components became evident, some years after the onset of cutaneous vasculitis.

Early diagnosis of APS 1 and ongoing regular surveillance, including periodic evaluation of hormonal and biochemical parameters, are essential to allow the prevention of severe and life-threatening events (i.e. hypocalcaemia, adrenal crisis) [2]. Therefore, although clinical components of APS 1 usually result from organ-specific autoimmune targeting, our case suggests that APS 1 should be also suspected in those cases presenting with immune-mediated non-organ-specific diseases, such as cutaneous vasculitis.

Recently, neutralizing autoantibodies against type 1 interferons (IFN) (IFN-α and IFN-ω) have been found to strictly correlate with *AIRE* deficiency, regardless of the genotype, thus leading to consider these autoantibodies as a precocious diagnostic tool for APS 1, even in the absence of the typical clinical picture or organ-specific autoantibodies [24,25]. However, it must be considered that molecular analysis, despite more expensive than autoantibody assay, may be more easily accessible for many laboratories. In our case, assay for anti-IFN antibodies was not available at the onset of the disease in our patient, but the above antibodies were found to be positive at the age of 9 years. Therefore, we can only speculate that their positivity could have been useful for an earlier diagnosis.

In addition, our case confirms the importance of the surveillance in searching for other sentinel autoantibodies, mainly those against the adrenal cortex and the related antigen targets, which show high predictive value for the occurrence of the related clinical component [26,27].

## Conclusions

In conclusion, although causal relationship between APS 1 and skin rashes or cutaneous vasculitis is still unclear, there is evidence pointing toward a close link between these conditions. Based on this hypothesis, our case provides further evidence that several minor or rare autoimmune or immune-mediated organ- and non-organ-specific diseases, such as cutaneous vasculitis, may dominate the initial clinical picture of APS 1, even for years before the development of the classic components of the disease. Therefore, although the diagnostic criteria of APS 1 remain valid, an atypical phenotype of APS 1 should be suspected in the presence of an early non-specific immune-mediated manifestation. Given the high specificity for APS 1 of anti-IFN autoantibodies, their evaluation may be a simple diagnostic tool for an early diagnosis. Finally, a regular check for hormonal, biochemical abnormalities, and organ-specific antibodies should be performed during follow-up, in order to recognize immune-mediated organs damage at an early stage, thus allowing to prevent potentially life-threatening events, such as hypocalcemia and adrenal failure.

## Consent

Written informed consent was obtained from the patient's parents for publication of this Case report. A copy of the written consent is available for review by the Editor of this journal.

### Abbreviations

APS 1: Autoimmune polyendocrine syndrome type 1; APECED: Autoimmune polyendocrinopathy candidiasis-ectodermal-dystrophy; *AIRE*: Autoimmune regulator gene; CMC: Chronic mucocoutaneous candidiasis; HPT: Chronic idiopathic hypoparathyroidism; AD: Addison disease; ANA: Anti-nuclear antibodies; p- and c- ANCA: Perinuclear and cytoplasmic anti-neutrophil cytoplasmic antibodies; FT4: Free-T4; ALPS: Autoimmune lymphoproliferative syndrome; IFN: Interferons.

### Competing interests

The authors declare that they have not competing interests.

### Authors' contributions

All authors contributed to conception and design, and gave final approval of the version to be published. In detail: NI drafted the manuscript; DC, CP and MS participated to its design and coordination and helped to draft the manuscript; DC, CP, MC, AE, EC and MS have been involved in revising the manuscript critically for important intellectual contents.

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# Chapter VIII

# Conclusive Remarks

PIDs are rare inborn errors of immunity whose expressivity and penetrance vary widely, even among family members with the same specific mutation, suggesting that genetic, epigenetic, and/or environmental factors may contribute to the clinical disease phenotype.

Early diagnosis of PID is useful in order to prevent disease-associated morbidity and mortality and improve QoL. However, to date the diagnosis of a specific PID based on the analysis of the phenotype remains difficult and a considerable delay, between the onset of the symptoms and diagnosis, is not rare. In the last years, the availability of NGS technologies is revolutionizing the discovery of genes in which variants can cause rare inherited diseases characterized by strong clinical and genetic heterogeneity, as PIDs.

In this PhD thesis, I pointed my attention on the evaluation of the potential benefit of very low dosage of betamethasone on neurological symptoms and Qol of patients affected with Ataxia-Telangiectasia, in the perspective of an occasional usage of the drug, thus preventing the occurrence of side effects. However, the major challenge of this study will be represented, in the future, by the the identification of the potential site of action of steroids in A-T, which will open a new window of intervention in this so far non-curable disease.

Furthermore, a better definition of the clinical and functional phenotype of patients with complex forms of PIDs, with attention to recent discovered gene, has been performed. Through NGS technologies, we have tried to understand the link between newly identified genes and the specific functional abnormalities resulting therefrom. This approach is essential for the implementation of new approaches for the clinical management of such patients and the development of precision medicine. NGS may provide a molecular diagnosis where previously the patient was unclassified, and thus may help in the identification of definitive therapeutic options, such as hematopoietic stem cell transplantation. On the other hand, detailed phenotypical data need to be validated and applied to the analysis steps to correlate disease-causing and disease-associated variants.

A further project described in this thesis concerns the evaluation of the pathogenetic mechanisms, including the evaluation of cellular response to DNA injury, in patients with increased IgM levels, impaired B-cell homeostasis and high

incidence of lymphoproliferation. NGS technologies revealed in two of them mutations in the PIK3R1 and ITPKB genes, implicated in T- and B-cell development and survival. This study highlights the possible role of polyclonal hyper IgM as biomarker of immune dysregulation and cancer susceptibility.

Taken together, our findings strengthen the importance of a global approach to the pediatric patient with PIDs.

Future research should hopefully extend our results, in order to verify their applicability and efficacy in improving health outcomes in patients with complex inherited disorders of immune system.

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Management of pediatric patients with Primary Immunodeficiencies;
Characterization of new forms of PIDs, with particular emphasis on Hyper IgM syndromes and Severe Combined Immunodeficiency (SCID) by Next Generation Sequencing Technology;
Definition of new therapeutic strategies for patients with Ataxia-Teleangiectasia;
Clinical and immunological characterization of patients with 22q11.2DS and other well defined PIDs

**Awards**

Best e-poster with the abstract "Altered fusion process between autophagosomes and lysosomes in lymphocytes from patients affected with Ataxia Telangiectasia (A-T)"

at the 17th Biennal Meeting of the European Society of Primary Immunodeficiency (ESID), Barcellona, September 2016.

**Invited as a speaker**

"La sindrome da delezione 22q11.2". Corso Immunologia per il Pediatra. Naples, March 06, 2015.

"Ascessi cutanei: quando sospettare un'immunodeficienza". National Congress -Pediatria a Napoli: dal caso clinico alle linee guida, Naples, Jenuary 21-23, 2016.

"Come migliorare la qualità delle cure: che cos'è un PDTA e a che serve". Giornata di incontro medici-famiglie dei pazienti affetti da Immunodeficienza primitiva della regione Campania. Naples, March 2016 con

"Manifestazioni ORL della sindrome Del22". Giornate IPINET 2016. Letojanni, May 27-28, 2016.

"Protocollo SCID/CIDs". Giornate IPINET 2016. Letojanni, May 27-28, 2016.

"Altered fusion process between autophagosomes and lysosomes in lymphocytes from patients affected with Ataxia Telangiectasia (A-T)", e-Poster presentation, ESID 2016 Biennal Meeting, Barcelona, Spain.

"Raccomandazioni sulle Immunodeficienze Severe Combinate: focus su segni di presentazione e storia naturale". Giornate IPINET 2017. Turin May 25-26, 2017.

**Professional affiliations**

Member of the European Society for Primary Immunodeficiency

**List of publications in the years 2014-2017**

- Improda N, Capalbo D, **Cirillo E**, Cerbone M, Esposito A, Pignata C, Salerno MC. Cutaneous vasculitis in patients with autoimmune polyendocrine sindrome type 1: report of a case and brief review of the literature. BMC Pediatrics, 14:272, 2014. doi: 10.1186/1471-2431-14-272.

- D'Assante R, Fusco A, Palamaro L, Giardino G, Gallo V, **Cirillo E**, Pignata C. Unraveling the link between Ectodermal disorders and Primary Immunodeficiencies. Int Rev Immunol, 2015. 10.3109/08830185.2015.1010724.

**- Cirillo E,** Giardino G, Gallo V, D'Assante R, Grasso F, Romano R, Lillo CD, Galasso G, Pignata C. Severe combined immunodeficiency-an update. Ann N Y Acad Sci, 2015. doi: 10.1111/nyas.12849.

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- Giardino G, Somma D, **Cirillo E**, Ruggiero G, Terrazzano G, Rubino V, Ursini V, Vairo D, Badolato R, Carsetti R, Leonardi A, Puel A, Pignata C. Novel STAT1 gain of function mutation and suppurative infections. Pediatr Allergy Immunol. doi: 10.1111/pai.12496.

- De Martino L, Capalbo D, Improda N, Lorello P, Ungaro C, Di Mase R, **Cirillo E**, Pignata C, Salerno MC. Novel findings into AIRE genetics and functioning: clinical implications. Front Pediatr 4:107. doi.org/10.3389/fped.2016.00107

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- Gallo V, Dotta L, Giardino G, **Cirillo E**, Lougaris V, D'Assante R, Prandini A, Consolini R, Farrow EG, Thiffault I, Saunders C, Leonardi A, Plebani A, Badolato R, Pignata C. Diagnostics of primary immunodeficiencies through Next Generation Sequencing. Front Immunol. 2016 Nov 7;7:466. eCollection 2016.

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**Abstracts and Communications**

- **Cirillo E**, Gallo V, Giardino G, Galasso G, Romano R, D'Assante R, gensio R, Baldini A, Nitsch L, Pignata C. DiGeorge–like sindrome in a child with a 3p12.3 deletion invloving MIRNA-4273 born to a diabetic mother. ESID 2014 Biennal Meeting, Prague, Czech Republic.

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- Giardino G, **Cirillo E,** Gallo V, D'Assante R, Paciolla M, Ruggiero G, Ursini MV, Carsetti R, Puel A, Pignata C. Chronic mucocutaneous candidiasis, recurrent herpetic infections and suppurative eyelid infections n a patient carrying a novel gain of function mutation in the STAT1 DNA-Binding domain. ESID 2014 Biennal Meeting, Prague, Czech Republic.

- Giardino G, Naddei R, **Cirillo E**, Gallo V, Esposito T, Fusco F, Quinti I, Urini MV, Carsetti R, Pignata C. TLR9 signaling in patients with Ectodermal Displasia and immunodeficiency associated with nuclear factor essential modulator (NEMO) mutations. ESID 2014 Biennal Meeting, Prague, Czech Republic.

- Giardino G, Gallo V, **Cirillo E**, Prencipe R, Di Giacomo C, Palamaro L, D'assante R, Lougaris V, Saunders CJ, Pignata C. Late onset combined immunodeficiency of unknown genetic origin presenting with persistent ebv-viremia, colitis and pulmonary aspergillosis. ESID 2016 Biennal Meeting, Barcelona, Spain.

- Moschese V, Sgrulletti M, Graziani S, Chini L, Tonti G, **Cirillo E**, Pignata C, Specchia F. Clinical characterization and prediction of outcome in patients with selective IgA deficiency. ESID 2016 Biennal Meeting, Barcelona, Spain.

-**Cirillo E**, Cruoglio L, Maiorino A, Gallo V, Giardino G, Vecchio O, Motta S, Pignata C. Otolaryngologic disorders in 22q11.2 deletion syndrome patients. ESID 2016 Biennal Meeting, Barcelona, Spain.

-Romano R, Gallo V, Giardino G, Galasso G, Maiorino A, Pignata C, **Cirillo E**. A broncho-vascular anomaly in a patient with DiGeorge syndrome. ESID 2016 Biennal Meeting, Barcelona, Spain

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-D'Assante R, Palamaro L, Prencipe MR, Giardino G, **Cirillo E,** Gallo V, Bianchino G, Grieco V, Pignata C. Altered fusion process between autophagosomes and lysosomes in lymphocytes from patients affected with Ataxia Telangiectasia (A-T). ESID 2016 Biennal Meeting, Barcelona, Spain

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