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PhD Thesis

Novel strategies in the approach to primary immunodeficiencies to discover new pathogenic mechanisms and complex clinical phenotypes

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BACKGROUND AND AIM

Primary immunodeficiency disorders (PIDs) are a group of inherited disorders of the immune system, resulting in an increased rate and severity of infection. Malignancy and immune dysregulation, the latter resulting in autoimmune disease and aberrant inflammatory responses may also feature these syndromes (1). PIDs differ from secondary immunodeficiencies occurring during certain viral infections, after immunosuppression, during treatment of systemic autoimmune disease, or in association with cancer chemotherapy (1).

The overall prevalence of PIDs is about 1:2000 live births (2, 3) with an estimated prevalence of the severe forms of PIDs of 1:58000 live birth (4). The male/female ratio of PIDDs is approximately 5:1 in infants and children but approaches 1:1 in adults (2, 5). PIDs are classified according to mechanistic and clinical descriptive characteristics. In particular, according to the mechanistic characteristics, they may be divided in defects of innate immunity, disorders of immune dysregulation, autoinflammatory syndromes, phagocyte and complement system defects, or defect of adaptive immunity. The last group can be further subdivided into humoral deficiencies, and combined deficiencies affecting both humoral and cellular mechanisms. Recently, anticytokine autoantibodies have been identified in the pathophysiology of some Mendelian PIDs. PIDs characterized by antibody deficiency are the most frequent group, accounting for approximately half of all patients with PIDs (2, 6).

Increased susceptibility to infections represent the main clinical feature of PIDs. The organ systems involved and the typical pathogens vary with the specific type of immune

defect (7, 8). Other features include autoimmune disease and malignancy, which may be often observed in a variety of immunodeficiencies (9, 10). Hypersensitivity to environmental and/or food allergens, may also feature a variety of PIDs (11).

A careful identification of the foci of infections, of the organisms, and of the response to treatment are necessary to point the diagnosis through a specific PID. Any other conditions that might predispose to infection, including anatomic defects, allergy, and metabolic disorders, should be also ruled out (12).

The clinical presentation usually guide the initial evaluation with a stepwise approach (screening tests followed by advanced tests) that ensures an efficient evaluation of mechanisms of immune dysfunction underlying the clinical presentation (2, 13). In some case, diagnosis may be difficult and may require costly sophisticated tests. First line laboratory assessment includes evaluation of serum immunoglobulin levels and leukocyte and lymphocyte subpopulations, and evaluation of the specific immune response including responses against vaccine antigens, or responses to natural exposure or infections.

According to the severity of clinical manifestations combined immunodeficiencies are classified in severe combined immunodeficiency (SCID) or combined ID with a ‘‘less severe’’ clinical presentation (14). Patients with SCID show a complete absence of specific immunity and are prone to develop infections caused by the entire range of possible pathogens, including opportunistic organisms (15-17). SCID is a life threatning condition and diagnosis is an urgent medical condition since if not treated these infants can succumb to severe infection at any time. Definitive hematopoietic stem cell therapy (HSCT) should be

performed as soon as possible (18-22). A variety of additional genetic defects leading to impairment of T- and B-cell function have been described, including hyper-IgM Wiskott-Aldrich, DiGeorge, ataxia-telangiectasia, hyper-IgE syndromes, and others. Many of these diseases are characterized by peculiar clinical features that might influence or guide the diagnostic approach.

The principal clinical manifestations of humoral immunodeficiency are recurrent bacterial infections of the upper and lower respiratory tract (23, 24).

The disorders of immune dysregulation include the hemophagocytic syndromes, syndromes with autoimmunity and hypersensitivity, and lymphoproliferation. The hemophagocytic syndromes are characterized by fulminant acute presentations triggered by viral infections (25, 26). These patients usually require aggressive chemotherapy followed by HSCT to prevent immediate fatality (25, 26).

Phagocytic cell defects can present with severe pyogenic bacterial and fungal infections of the respiratory tract, skin, and viscera and gingivostomatitis. Laboratory evaluation might show neutropenia, normal neutrophil numbers, or marked neutrophilia (mainly in cellular adhesion defects) (27, 28).

Disorders of innate immunity are rare and include defects of Toll-like receptor (TLRs) signaling, such as nuclear factor kB essential modulator (NEMO) syndrome, often exhibiting ectodermal dysplasia along with infection susceptibility with a narrow (eg, predominantly pyogenic bacteria or fungi) to a wide range of pathogens (29, 30). This category also includes

several defects associated with herpes simplex encephalitis and chronic mucocutaneous candidiasis (CMC) (31).

More than 200 distinct genetic disorders responsible of well-characterized PIDs have been identified to date (32). Alterations in such genes, implicated in immune function, underlie very complex phenotype. Early diagnosis of PID is useful in order to prevent significant disease-associated morbidity and mortality (33). However, to date the diagnosis of a specific PID based on the analysis of the individual clinical and/or immunological phenotype remains often difficult, and a considerable delay between the onset of symptoms and the time of diagnosis of PIDs is often reported. About the 40% of patients with these disorders do not reach a diagnosis until adulthood despite the presence of serious chronic health conditions prior to diagnosis, such as sinusitis, bronchitis, and pneumonia (34).

Thanks to the progress in molecular technologies, a remarkable improvement of the knowledge in the field of PIDs, concerning both their etiopathogenetic mechanisms and clinical features, has been observed in the last 20 years, leading to a complete revisitation of the classical paradigms of PIDs (35). PIDs, in fact, were initially thought to be confined to a few rare, familial, monogenic, recessive traits impairing the development or function of one or several leucocyte subsets and resulting in multiple, recurrent, opportunistic and fatal infections in infancy. A growing number of exceptions to each of these conventional qualifications have accumulated over the years, particularly over the last two decade (Table I) (35).

Table I: Multiple paradigm shifts in human primary immunodeficiencies (35).

Table 1 Multiple paradigm shifts in human primary immunodeficiencies

Primary immunodeficiencies	Conventional	Novel	Examples
Epidemiological levels			
Frequency	Rare	Common	*FUT2* mutations and norovirus
Occurrence	Familial	Sporadic	*UNC93B1* and *TLR3* mutations and HSE
Age at onset	Childhood	Adulthood	CVID
Prognosis	Spontaneously worsening	Spontaneously improving	IRAK-4 deficiency
Phenotype level			
Disease-defining clinical phenotypes	Opportunistic infections[a]	Other life-threatening infections, other phenotypes[b]	Crohn's disease and impaired inflammation
Number of phenotypes per patient (e.g. infectious agents)	High	Low (even single)	Properdin and *MAC* mutations and Neisseria
Number of episodes per patient (e.g. infectious episodes)	High	Low (even single)	IL-12p40 and IL-12Rβ1 deficiency
Disease-causing cellular phenotypes	Haematopoietic	Nonhaematopoietic	*EVER1* and *EVER2* mutations and HPV
Genotype level			
Mode of Mendelian inheritance	Autosomal and X –recessive	Autosomal dominant	*STAT1* mutations and mycobacteria
Clinical penetrance	Complete	Incomplete	*IFNGR1* mutations and mycobacteri
Disease-causing genes per patient	One (monogenic, Mendelian)	Several (oligogenic, major genes)	*PARK* and *LTA* and Leprosy
Mutations	Inherited from the parental genome	Inherited from the parental germline *de novo*, or somatic	*Fas* mutations and auto-immunity

[a]Infections occurring in patients with overt immunological abnormalities.
[b]Autoimmunity, allergy, virus-induced cancer, angioedema, granulomas, haemophagocytosis, autoinflammation, thrombotic microangiopathy.

Until 2010, the traditional approach to PIDs diagnosis has included Sanger sequencing of candidate genes, single nucleotide polymorphisms (SNPs), linkage analysis, and has involved an array of specialized functional tests including lymphocyte proliferation and cytotoxicity assays, flow cytometry, measurement of serum immunoglobulin levels, neutrophil function tests. With this traditional approach a definitive diagnosis is not possible in many cases (34).

In the very last years, genetic identification of immunodeficiency syndromes has become more efficient with the availability of whole-exome sequencing (WES), expediting the identification of relevant genes and complementing traditional linkage analysis and homozygosity mapping (Table II) (36, 37). Only in the last two years, 34 new gene defects have been identified (Table II and III) (32, 38).

Table II: Selected key advances in basic and clinic immunology in 2013 (38)

PIDs: Mechanisms of disease
• High levels of nonglycosylated IgA contribute to nephropathy in patients with WAS.
• TCR ligation causes WASP degradation, controlling T-cell activation.
• Defective STAT3 signaling impairs mast cell degranulation and CD8 T-cell activation.
• Antibody deficiency in patients with AT is caused by disturbed B- and T-cell homeostasis and reduced immune repertoire diversity.
• *TACI* mutations impair B-cell function in healthy mutation carriers.
PIDs: New genes
• *CARD11* inactivation is associated with SCID.
• *MALT1* mutation causes CIDs.
• *TTC7A* mutations are associated with multiple atresia with CID.
• Combined *DOCK8* and *CLECL7A* mutations cause immunodeficiency.
PIDs: New phenotypes
• Adult-onset idiopathic T-cell lymphopenia and early-onset autoimmunity might be due to a heterozygous *RAG1* mutation.
• Hypomorphic *JAK3* mutations might allow maternal T-cell engraftment and development of hypofunctional NK cells.
• CORO1A deficiency might present with immunodeficiency and EBV-associated B-cell lymphoproliferation.
PIDs: Diagnosis
• Newborn screening in California established the incidence of SCID at 1 in 66,250 live births.
• Patients with MHC class II deficiency and late-onset ADA-SCID might have normal TREC levels at birth.
PIDs: Treatment
• Patients with ADA-SCID on PEG-ADA present with increased frequency of allergic disease.
• IL-10R–deficient patients significantly decrease symptoms after HSCT.
• G-CSF therapy for isolated CMC might result in complete remission.

Table III: Novel gene defects as a cause of primary immunodeficiencies identified in 2014 (modified by (32))

Gene	Inheritance	Immunodeficiency syndrome
PGM3	Autosomal recessive	Atopy, autoimmunity, frequent infection, developmental delay
IL21	Autosomal recessive	Inflammatory bowel disease, hypogammaglobulinemia
CTLA4	Autosomal dominant	CVID, ALPS
PIK3R1	Autosomal recessive	Hypogammaglobulinemia, frequent infections
MAP3K14	Autosomal recessive	Combined immunodeficiency
BCL10	Autosomal recessive	Combined immunodeficiency
CTPS1	Autosomal recessive	Combined immunodeficiency
JAGN1	Autosomal recessive	Congenital neutropenia
TMEM173	Autosomal dominant	Skin and pulmonary vasculopathy, early-onset inflammation
NLRC4	Autosomal dominant	Fever, rash, arthralgia induced by cold Periodic fever, macrophage activation syndrome
CECR1	Autosomal recessive	Polyarteritis nodosa and hypogammaglobulinemia Livedo reticularis and neurovascular disease (Sneddon syndrome)
TRNT1	Autosomal recessive	SIFD

In this context, this thesis will be focused on novel insight into diagnostic approach to PIDs and on the definition of novel clinical and laboratory features of new and old PIDs. Moreover, novelties in the fields of inheritance of PIDs will be discussed. This thesis reports the results obtained during my PhD course in "Human Reproduction, Development and Growth" (XXVIII Cycle) from 2013 to 2016. During the past 3 years, my research has been focused in the study of the following lines of research:

- Characterization of novel aspect of the pathogenesis of already known immunodeficiency, diagnosed conventionally or through Next Generation Sequencing (NGS). In particular, my research was focused on the study of the role of Myeloid differentiation factor 88 (MyD88) deficiency, identified through Targeted NGS, in the pathogenesis of the immunological and clinical features observed in a patient who had an atypical presentation characterized by chronic Yersiniosis and granuolomatous lymphadenitis;
- Better characterization of the clinical phenotype of a complex syndrome caused by STAT1 gain of function mutation, whose hallmark is autosomal dominant Chronic Mucocutaneus Candidiasis (CMCD) and definition of the role of such mutations in the pathogenesis of clinical manifestations associated to the syndrome;
- Definition of the role of T-independent B-cell immunity in susceptibility to infections from encapsulated bacteria in Hypoidrotic Ectodermal Dysplasia with immununodeficiency (HED-ID);

- Characterization of skin and skin annexa abnormalities associated to PIDs, which represent alarm signs that should lead the clinician to consider a deeper immunological assessment;
- Study of the functional role of FOXN1 transcription factor in the T-cell ontogeny;
- Rare genetic syndrome involving immune system paying a particular attention to SCID, hemophagocytic lymphoistiocitosis (HLH) and Di George Syndrome (DGS).

CHAPTER I

"Targeted Next Generation Sequencing (TNGS): a powerfull tool for a rapid diagnosis of PIDs"

PIDs represent a vast group of monogenic diseases including more than 250 genetically defined diseases (39). Most of the genetic defect have been identified in recent years. The rapid advance of the knowledge in the field of functional immunological studies and genetic diagnostic tools, contributed to this extraordinary rapid evolution (32, 38). However, despite the breadth of current knowledge, the early genetic diagnosis underlying PIDs, still remains a difficult task with the traditional stepwise diagnostic approach, based on functional studies followed by Sanger sequencing (34). Early diagnosis in severe forms of PIDs is crucial to establish a proper treatment and improve the overall outcome (34). Moreover, diagnosis at the molecular level is desirable to: 1. establish unequivocal diagnosis, 2. permit accurate genetic counseling, 3. allow planning of future pregnancies or their outcomes, 4. better define genotype/phenotype associations, and 5. identify candidates for gene-specific therapies.

The traditional approach to PIDs, often represents a time consuming strategy, which require the sequencing of multiple genes by the classic Sanger method, and may lead to delayed diagnosis. In fact, different mutations in the same gene can lead to different clinical phenotypes (32, 38). For example, a specific recombination-activating gene 1 (RAG1) variant protein with partial recombinant activity might produce Omenn syndrome, a leaky SCID or a Common Variable Immunodeficiency (CVID) phenotype. Moreover, defect in

different genes may lead to very similar clinical phenotype. Furthermore, not all the PIDs causing genes are known and many PIDs represent extremely rare conditions.

In this context, NGS technology may represent a powerful diagnostic tool for the rapid detection of the genetic alteration, particularly in complex cases (40). In the field of PIDs, targeted sequencing approach may be tailored on a peculiar functional alteration, that characterizes a specific disorder, allowing an easier management of the data sets, along with lower cost, than the most complex WES analysis (41-43).

The NGS approach represent a very powerful tool for the rapid gene identification of typical phenotypes, for the identification of new genetic defects associated to classical phenotypes, for the definitions of new clinical presentation of a known gene alterations and for the identification of new genetic defects associated to new clinical phenotypes.

1.1 Targeted next generation sequencing revealed MYD88 deficiency in a child with chronic Yersiniosis and granulomatous

MyD88 deficiency is a very rare PIDs affecting the innate immunity, inherited as an autosomal recessive trait (44, 45). MyD88 is a cytosolic adapter molecule, linking TLRs and interleukin-1 receptors (IL-1Rs) to the IRAK complex. MyD88 and IRAK-4 deficiencies are responsible for very similar clinical phenotypes (45) (Figure 1).

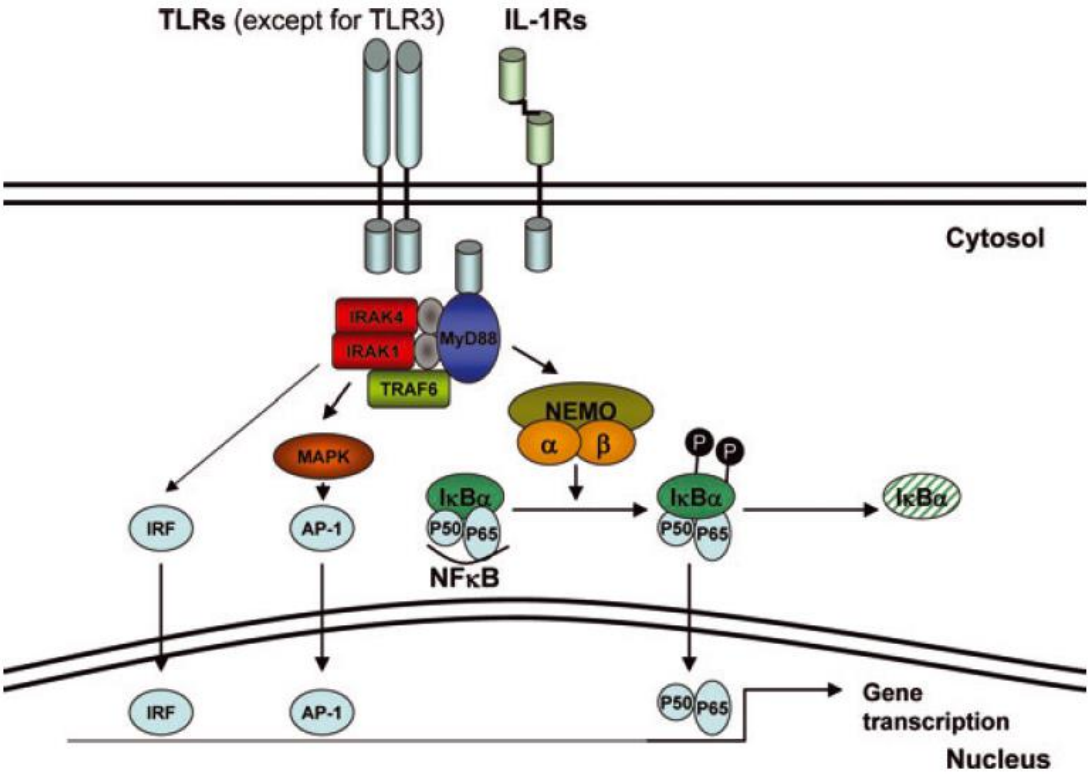


Figure 1. MyD88- and IRAK-4-signaling pathways (46).

PBMC from MyD88- and IRAK-4-deficient patients show impaired responses to the stimulation with TLR ligands (other than TLR3 ligands) (47, 48) and IL-1R agonists (44, 45). The MyD88- pathway is implicated in the synthesis of inflammatory cytokines (IL-1β, IL-6, IL-8, tumor necrosis factor [TNF]-α, interferon [IFN]-α/β, and IFN-λ) (49).

MyD88 deficiency is characterized by increased susceptibility to invasive bacterial disease (InvBD) caused by Streptococcus pneumoniae (S. pneumoniae) and Staphylococcus aureus (S. Aureus), and to non-invasive bacterial disease (NInvBD) caused by S. Aureus and Pseudomonas aeruginosa (P. Aeruginosa) (50). Recurrent invasive pneumococcal diseases

represent a hallmark of this PID (45). Due to the very weak inflammatory signs, which usually appear late, infections in Myd88 deficiency may be very difficult to diagnose. NInvBD usually involve to the skin (S. Aureus) and the upper respiratory tract (P. aeruginosa). By contrast, during InvBD several sites are involved, and abscesses of inner organs, lymph nodes and saliva glands, meningitis, and septicemia may be frequently observed. Involvement of gastrointestinal and urogenital system is very rare (50). On the other side, MyD88-deficient patients display a normal resistance to common fungi, parasites, viruses, and to a large fraction of bacteria (46). Even in cases of severe infections, clinical and laboratory signs of inflammation usually develop very slowly and a frank neutropenia may develop from the onset of infection as consequence of impaired polymorphonuclear neutrophil mobilization, perhaps secondary to the lack of IL-8 production (50). MyD88 deficiency may be life-threatening in infancy and childhood, with a mortality rate of about 38%. Thanks to the development of adaptive antigen-specific T- and B-lymphocyte responses, infections in Myd88 deficient patients are rarer with age, and no death has been recorded after the age of 8 years and no invasive infection after the age of 14 years, even when antibiotic prophylaxis and IgG replacement therapy are discontinued (50).

In this paper published as *Letter to the Editor* on *Journal of Allergy and Clinical Immunology* we report on the case of a 2-year-old female born to consanguineous parents of Rom ethnia. Since the age of 7 months the patient experienced chronic Yersiniosis, recurrent granulomatous lymphadenitis, and episodic neutropenia. TNGS panel, with customized analysis for immunodeficiency genes, lead to the identification of a homozygous in-frame deletion, p.Glu66del in the *MYD88* gene.

Letter to the Editor

Targeted next-generation sequencing revealed *MYD88* deficiency in a child with chronic yersiniosis and granulomatous lymphadenitis

To the Editor:

Yersiniosis is a food-borne illness, usually self-limited. Severe clinical courses may occur in chronic conditions, particularly in immunocompromised individuals.[1]

Here, we report on the case of a 2-year-old girl born to consanguineous parents of Roma descent (a traditionally itinerant ethnic group living mostly in Europe and the Americas, who originate from Northern India), with chronic yersiniosis, recurrent granulomatous lymphadenitis, and episodicneutropenia. Using a targeted next-generation sequencing panel for immunodeficiency genes, a homozygous in-frame deletion in the *MYD88* gene was found. MyD88 is a key downstream adapter for most Toll-like receptors (TLRs) and IL-1 receptors (IL-1Rs).[2] *MYD88* deficiency has been associated with life-threatening and recurrent pyogenic bacterial infections, including invasive pneumococcal disease.[3,4]

The child was born at term from a pregnancy complicated by maternal syphilis, which required a postnatal treatment with benzathine penicillin. During the treatment, she developed a severe neutropenia, from which she spontaneously recovered. A congenital syphilis infection was excluded by serological, blood culture, and cerebrospinal fluid examination, cerebral ultrasound, and long bone x-ray image. Familial history was significant for 3 brothers who died in childhood of infectious diseases, 2 of meningitidis and sinusitis and 1 of enteritis (Fig 1, *A*). No further information was available about the brothers' clinical history. At the age of 7 months, she developed an episode of upper left arm edema associated with fever followed by an intestinal occlusion. Exploratory laparotomy revealed severe stenosis of the ileal tract from 10 to 40 cm proximal to the ileocecal valve, associated with mesenteric adenitis. Intestinal wall histology revealed conspicuous infiltration of bacilli (Fig 2, *C* and *D*), vast areas of marginal necrosis, full-thickness massive edema, and inflammatory infiltrates (Fig 2, *E* and *F*). Cultures of stool and of the bowel wall were negative for bacteria, parasites, and mycobacterium tuberculosis. Cytomegalovirus, human immunodeficiency virus (HIV1/HIV2), Salmonella/Brucella, and *Treponema pallidum* (Rapid plasma reagin/treponema pallidum hemagglutination assay, *T pallidum* IgM/IgG) were also excluded by serological examinations. At the age of 13 months, she developed a new episode characterized by fever, abdominal pain, diarrhea, and vomiting. On this occasion, blood culture grew *Yersinia*, which required treatment with amoxicilline/clavulanic acid.

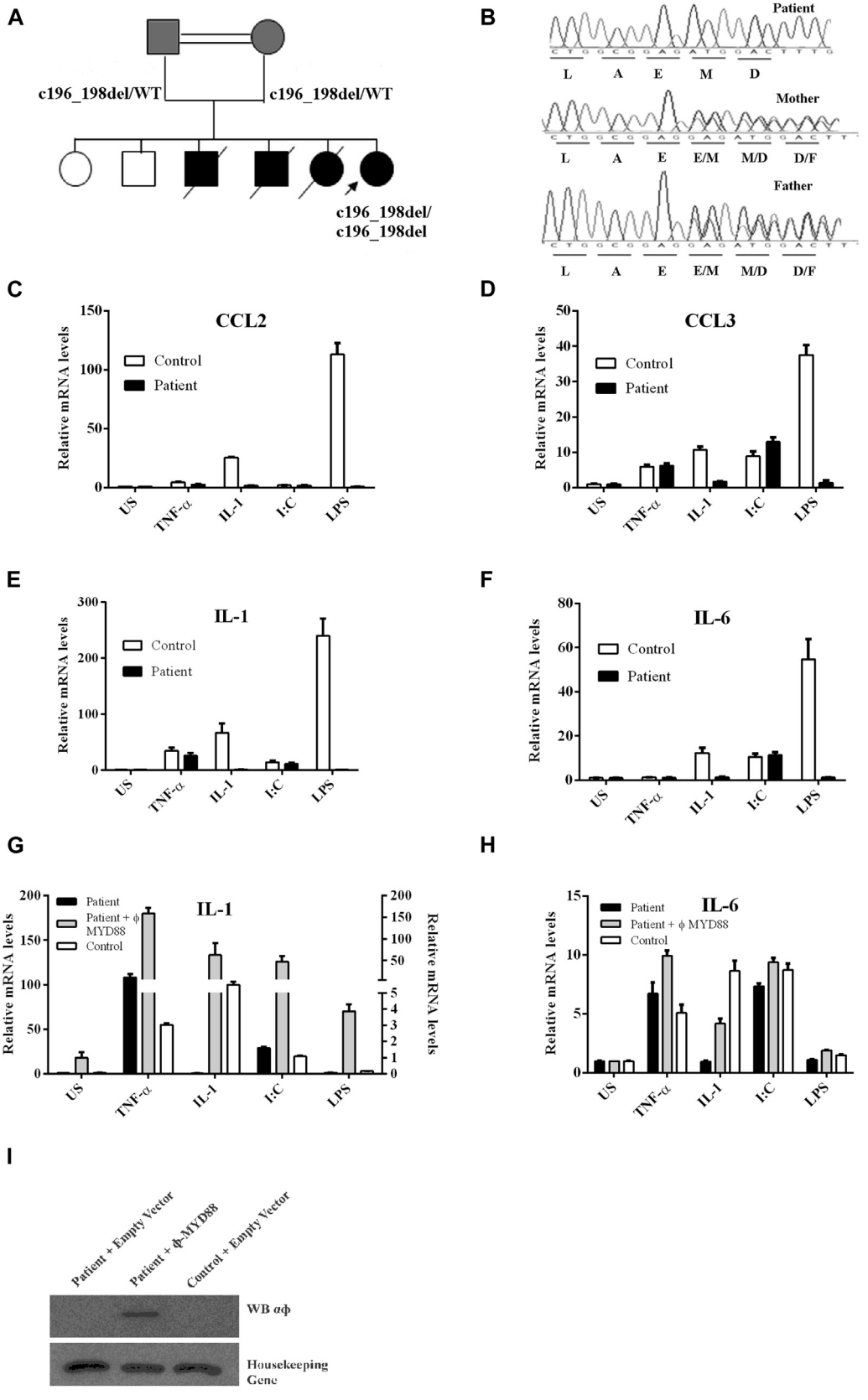
At the age of 8 months, she developed axillary (Fig 2, *A* and *B*) and epitrochlear suppurative lymphadenitis of the left arm. The histological examination of the 5-cm diameter cold axillary colliquative lymphadenitis revealed a mixed inflammatory reaction characterized by T and B lymphocytes, plasma cells, and eosinophils. Histiocytes, mixed with multinucleated giant cells arranged in a palisading pattern, were observed, consistent with a granulomatous inflammatory process. Cultures from the purulent exudate grew *Staphylococcus aureus* sensitive to amoxicilline/clavulanic acid. The patient never experienced eczema or any other cutaneous lesion, and abscesses developed on an otherwise healthy skin. Laboratory examinations revealed microcytic hypocromic anemia (hemoglobin, 8.9 g/dL; mean corpuscular volume, 60 fl; mean corpuscular hemoglobin, 18 pg), eosinophilia (750 cells/μL), and normal inflammatory index (C-reactive protein, 0.1 mg/dL; ferritine, 73.8 ng/mL). After unsuccessful intravenous antibiotic therapy, surgical drainage was required. Prophylactic treatment with cotrimoxazole and amoxicilline/clavulanic acid was then given for potential recurrence of *S aureus* lymphadenitis.

An immunodeficiency was suspected on the basis of the clinical features and familial history. Other genetic disorders, not directly involving the immune system, were ruled out by the clinical history and routine laboratory examinations. First-level immunological examinations revealed normal IgG, IgM, and IgA levels, increased IgE levels (1190 IU/mL), normal levels of standard lymphocyte subpopulations ($CD3^+$ 57%, $CD4^+$ 41%, $CD8^+$ 12%, $CD56^+$ 14%, $CD19^+$ 19%, $CD3^+HLA^-DR$ 3%) (for absolute numbers, see Table E1 in this article's Online Repository at www.jacionline.org) and of adhesion molecules (absolute monocyte number was 440 cells/μL and CD11a/CD18, CD11b/CD18, and CD11c/CD18 on the leucocyte gate was 99%). Specific antibodies were detectable, and the proliferative response to mitogens was normal. The study of the response to Prevenar13 revealed an impaired IgM, but not IgA and IgG, response against pneumococcal polysaccharides, as previously reported by Maglione et al.[5] This finding, associated with the evidence of a low titer of isohemagglutinins, suggests an impaired response against T-independent polysaccharidic antigens (see Table E2 in this article's Online Repository at www.jacionline.org). Nitroblue tetrazolium chloride and dihydrorhodamine 123 assays ruled out the chronic granulomatous disease. AT+B+NK+ combined immunodeficiency due to hypomorphic *RAG1/2* mutation, consistent with the presence of granulomatous inflammation of skin, was excluded by molecular analysis. Hyper-IgE syndrome score was ruled out through the molecular analysis of *STAT3*.

At the age of 11 months, she again developed a severe neutropenia, which eventually required G-CSF treatment. Congenital neutropenia was ruled out with negative *ELANE* gene sequencing.

After written informed consent from the parents for molecular genetic studies, a targeted next-generation sequencing panel (TaGSCANv.2: Targeted Gene Sequencing and Custom Analysis), including 572 genes and customized for 55 immunodeficiency genes, was run (see the Methods section and Table E3 in this article's Online Repository at www.jacionline.org).[6] A homozygous known pathogenic in-frame deletion, c.196_198del GGA (p.Glu66del; also referred to as E65del and E52del[3,7,8]), was identified in exon 1 of the *MYD88* gene. This variant has been previously reported in several affected families, at least 2 of which were of Rom descent with consanguineous parents. This deletion removes a single conserved glutamic acid residue in the Death domain, resulting in greatly diminished protein levels.[7] Sanger sequencing was used to verify the finding and confirm carrier status of both parents (Fig 1, *A* and *B*).

We studied the effect of MYD88 deficiency in TLR and IL-1R responses of PBMCs from the patient. *Il-6*, *il-1*, *ccl2*, and *ccl3* mRNA induction following TLR4 or IL-1R agonists


A
c196_198del/WT
c196_198del/WT
c196_198del/
c196_198del
B
Patient
L A E M D
Mother
L A E E/M M/D D/F
Father
L A E E/M M/D D/F
C
CCL2
Relative mRNA levels
Control
Patient
US TNF-α IL-1 I:C LPS
D
CCL3
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Patient
US TNF-α IL-1 I:C LPS
E
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Relative mRNA levels
Control
Patient
US TNF-α IL-1 I:C LPS
F
IL-6
Relative mRNA levels
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US TNF-α IL-1 I:C LPS
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Relative mRNA levels
Patient
Patient + ϕ MYD88
Control
US TNF-α IL-1 I:C LPS
H
IL-6
Relative mRNA levels
Patient
Patient + ϕ MYD88
Control
US TNF-α IL-1 I:C LPS
I
Patient + Empty Vector
Patient + ϕ-MYD88
Control + Empty Vector
WB αϕ
Housekeeping Gene

was abolished, while they were normally induced after TNF-α and TLR3 stimulation (Fig 1, *C-F*). Fibroblasts from the MYD88-deficient child and a healthy control were transiently transfected with an expression vector encoding the wild-type *MYD88* gene. Cells from MYD88-deficient patient regained IL-1β and LPS responsiveness after transfection with the wild-type *MYD88* gene, as shown by the levels of IL-1 and IL6 production (Fig 1, *G* and *H*) (see the Methods section).

In *MYD88*-deficient patients, impaired polymorphonuclear neutrophil mobilization and/or frank neutropenia may occur from the onset of infection, presumably related to the lack of IL-8 production. Gastrointestinal infections are rare.[9] Moreover, TLRs play a key role in the immunological response to flagellated Gram-negative bacterium, thus explaining why the patient developed chronic yersiniosis.[10] Of note, *Yersinia enterocolitica* is able to deactivate TLR-induced signaling pathways, by cleaving Myd88, triggering apoptosis in macrophages.[11] This mechanism may increase Yersinia virulence in immunocompetent hosts also. In a recent article by von Bernuth et al,[12] MyD88-deficient mice were found to be susceptible to almost nearly all the microbes tested, including bacteria, viruses, protozoa, and fungi, highlighting the importance of TLR in the immune response to different pathogens. In contrast, patients with MyD88 or IRAK-4 deficiencies have been shown to be susceptible to invasive and noninvasive infections with only a few Gram-positive and Gram-negative bacteria, maybe as a consequence of the redundancy of the immune system. However, the predominance of Gram-positive bacteria in patients with MyD88 and IRAK-4 deficiencies may result, at least in part, from a limited exposition to the different antigens in certain geographic areas. The evidence of chronic yersiniosis in this patient expands the list of organisms that can lead to infections in subjects affected with this syndrome. Even though we were not able to grow *Yersinia* from the bowel wall, it was suspected as being responsible for the terminal ileitis and mesenteric adenitis on the basis of the histological staining indicative of a bacillus infection.

In this study, we reported on a patient affected with chronic yersiniosis, recurrent granulomatous lymphadenitis, and neutropenia associated with an homozygous p.Glu66del variant in *MYD88*. This finding was unexpected because Myd88 deficiency is characterized by high susceptibility to invasive bacterial disease caused by *Streptococcus pneumoniae* and *S aureus*, with invasive pneumococcal disease being the hallmark of the syndrome.[9] Noninvasive bacterial diseases caused by *S aureus* or *Pseudomonas aeruginosa* have also been described.[9] The atypical presentation of our patient, characterized by chronic yersiniosis and recurrent neutropenia in the absence of recurrent invasive pneumococcal disease, made the diagnosis difficult. In these contexts, targeted next-generation sequencing may represent a rapid and low-cost tool to achieve an early diagnosis.

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FIG 1. *MYD88* deficiency impairs TLR and IL-1R responses of PBMCs. **A,** Family pedigree. The proband is indicated with an *arrow*. Healthy persons are shown in white; deceased siblings are indicated by *line crossing*. **B,** Genomic sequence analysis of *MYD88* gene showing a homozygous in-frame deletion, c.196_198del GGA (p.Glu66del). Both parents are heterozygous for the same mutation. **C-F,** Real-time PCR analysis of the mRNA extracted from the patient's PBMCs stimulated with LPS, IL-1, TNF-α, and poly (I:C) (TLR3 ligand) revealed reduced levels of IL-6, IL-1, CCL2, and CCL3, as compared with the healthy control, in response to LPS and IL-1 stimulation, while they were normally induced after TNF-α and TLR3 stimulation. **G** and **H,** Fibroblasts from a healthy control and from the MYD88-deficient child, transiently transfected with an expression vector encoding wild-type (WT) MYD88. Cells from MyD88-deficient patient regained IL-1β and LPS responsiveness after transfection with the WT *MYD88* gene, as shown by the levels of IL-1 and IL6. Y-axis values, on the *right side*, are referred to LPS stimulation. **I,** Western blotting using anti–FLAG-specific antibody to verify the transfection of patients' fibroblasts with plasmids carrying WT *MYD88*.

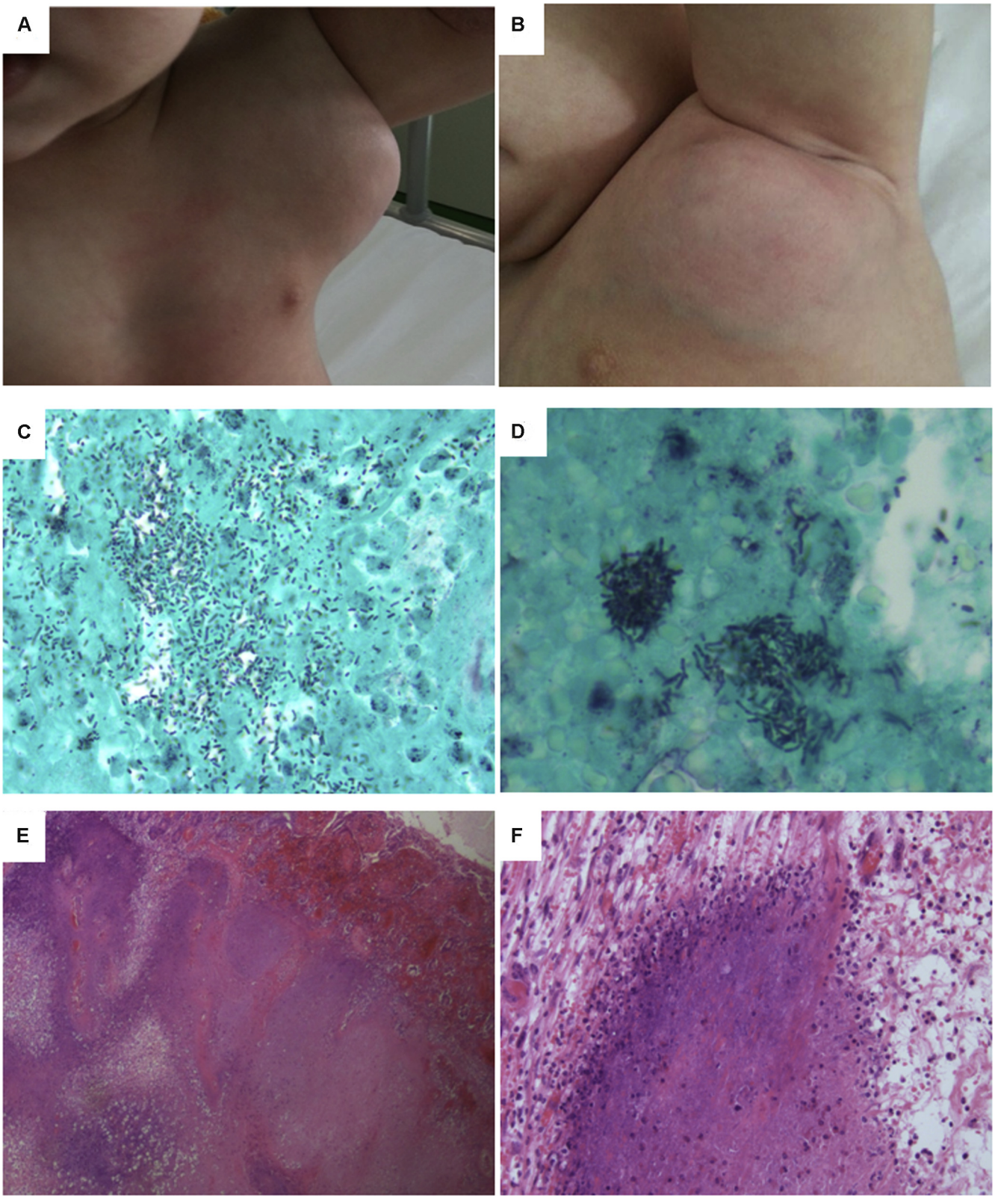

FIG 2. Clinical features. **A** and **B,** Axillary suppurative lymphadenitis of the left arm. **C** and **D,** Grocott stain revealed conspicuous infiltration of bacilli in the intestinal wall. **E** and **F,** Intestinal wall histology revealed vast areas of marginal necrosis, full-thickness massive edema, and inflammatory infiltrates.

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METHODS

Targeted next-generation panel, "TaGSCANv.2" (Targeted Gene Sequencing and Custom Analysis) was run, which includes 572 genes. Briefly, samples were prepared for sequencing using TruSight Rapid Capture with TruSight Inherited Disease Oligos (Illumina, San Diego, Calif); the MT genome is covered at 1000× by the addition of MT oligos during enrichment (Integrated DNA Technologies, Coralville, Iowa). Samples were sequenced to at least 2.5Gb on an Illumina MiSeq with TruSeq v3 reagents, yielding paired 250 nucleotide reads. Alignment, variant calling, and analysis were performed as previously described.[E1] In this case, analysis was customized for immunodeficiency, which limited the genes examined to 55 (see Table E3). Variant analysis was confined to coding and splice variants with a minor allele frequency of 1% or less in the Children's Mercy Hospital internal database. Two variants remained after filtering. One variant, in the *FERMT3* gene associated with leukocyte adhesion deficiency type III, was not pursued because the minor allele frequency is 0.96 in European Americans (National Heart, Lung, and Blood Institute database). No second variant was identified, and there was limited phenotypic overlap. In addition, a homozygous known pathogenic in-frame deletion, c.196_198del GGA (p.Glu66del; also referred to as E65del and E52del in the literature[E2-E4]), was identified in exon 1 of the *MYD88* gene. This variant has been previously reported in several affected families, at least 2 of which were of Roma descent with consanguineous parents. This deletion removes a single conserved glutamic acid residue in the Death domain, resulting in greatly diminished protein levels.[E4] Sanger sequencing was used to verify the finding and confirm carrier status of both parents (Fig 1, *A* and *B* in the text).

PBMCs were isolated from patients by density gradient centrifugation over Ficoll-Hypaque (Biochrom, Berlin, Germany). For the cytokine mRNA production, PBMCs were cultured in RPMI 1640 supplemented with 1% FBS, for 24 hours without stimulation and then stimulated with LPS 1 ng/mL, IL-1β 10 ng/mL, TNF-α 20 ng/mL, or poly(I:C) (TLR3 ligand) 25 μg/mL. The mRNA, extracted from the PBMCs, was analyzed by real-time PCR and standard procedures.

For the complementation of patients' fibroblasts with plasmids carrying wild-type MYD88, fibroblasts were incubated with a mixture containing Lipofectamine 2000 reagent (Invitrogen #52887) and plasmids, as previously reported.[E2] This complex was then removed and fibroblasts were incubated for 18 hours in Dulbecco modified Eagle medium supplemented with 10% FBS before stimulation. Cytokine mRNA production was assessed in fibroblasts cultured in Dulbecco modified Eagle medium supplemented with 10% FBS, incubated for 48 hours without stimulation and then with TNF-α, IL-1β, or poly(I:C) for 6 hours.

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TABLE E1. Absolute number of cell subpopulation

Cell subpopulation	Absolute number (reference value)
Leukocytes	8,960 (6,700-14,000)*
Lymphocytes	5,320 (1,800-18,700)
T cells ($CD3^+$)	3,032 (1,400-11,500)
CD4 cells	2,181 (1,000-7,200)
CD8 cells	638 (200-5,400)
CD3 HLA-DR	159
B cells ($CD19^+$)	1,010 (130-6,300)
Natural killer cells ($CD56^+CD3^-$)	745 (68-3,900)

NA, Not available.
*Normal reference value.[E5]

TABLE E2. Immunologic humoral evaluations of the patient

Laboratory evaluation	
Immunoglobulins (mg/dL)	
IgG	1180 (351-919)*
IgA	104 (10-85)
IgM	88 (38-204)
IgE (KU/L)	1190
Specific antibodies	
HBV antigen antibodies (mIU/mL)	143.9
HSV (IgG/IgM)	+/−
VZV (IgG/IgM)	+/−
CMV (IgG/IgM)	−/−
Rosolia (IgG/IgM)	+/−
Antibody response to pneumococcal polysaccharides (IgG/IgA/IgM)†	
IgG	8.0
IgA	27.2
IgM	0.7
Isohemagglutinins	Anti-A 1:32 Anti-B 1:4

CMV, Cytomegalovirus; *HBV*, hepatitis B virus; *HSV*, herpes simplex virus; *VZV*, varicella zoster virus.

*Normal reference values.

†Data indicate the fold-increase in the antibody titer after the booster. A positive response is defined as a threefold increase in the antibody titer.

TABLE E3. List of the 55 studied genes

ADA
AP3B1
ATM
BLM
BLOC1S6
BTK
CD19
CD247
CD3D
CD3E
CD3G
CD40LG
CFP
DCLRE1C
DOCK8
ERCC2
ERCC3
FERMT3
FOXN1
FOXP3
G6PC3
GTF2H5
ICOS
IFNGR1
IFNGR2
IKBKG
IL12B
IL12RB1
IL1RN
IL2RG
JAK3
LIG4
LYST
MPV17
MYD88
NHEJ1
ORAI1
PRF1
RAB27A
RAG1
RAG2
RMRP
SH2D1A
SLC35C1
SP110
STAT1
STIM1
STX11

Conclusive remarks

The atypical presentation of our patient, characterized by chronic Yersiniosis and recurrent neutropenia in the absence of recurrent invasive pneumococcal disease, made the diagnosis very difficult. In these contexts, NGS may represent a rapid and low-cost tool to achieve an early diagnosis.

1.2 Diagnostics of Primary Immunodeficiencies through targeted Next Generation Sequencing

Vera Gallo, Laura Dotta, Giuliana Giardino, Emilia Cirillo,Vassilios Lougaris, Roberta D'Assante, Alberto Prandini, Rita Consolini, Carol Saunders, Alessandro Plebani, Raffaele Badolato and Claudio Pignata

Introduction

NGS technology represent a powerful, cost-effective, first-line diagnostic tool for a rapid detection of a genetic alteration, particularly in complex cases (40). Through NGS technology sequence of million DNA fragments, ranging from selected genes to the entire human genome (whole genome sequencing) may be amplified simultaneously and with a high grade of accuracy, in a reasonably short time. The whole exome, which includes only the coding regions of the genome, accounts for about 2% of the entire genome, containing about 85% of genetic alterations associated to human diseases (51). Thus, WES may represent a valuable and rapid alternative to the whole genome sequencing when NGS fails to identify the genetic alteration.

In this study, we used NGS sequencing panel, including 571 targeted genes and WES to study a cohort of patients affected with complex clinical phenotypes, suggestive of severe inherited forms of PID of unknown genetic origin, in which a diagnosis was not obtained through current diagnostic procedures. In four patients we found alterations of genes associated to already genetically defined PIDs. In four patient alterations of already known PID genes, were associated with unusual clinical phenotypes. Functional and molecular studies confirmed the correlation between the genetic alteration and the alteration of the immunological function. In 8 patients we identified a total 22 gene variants in genes implicated in the immune response but not previously associated to any known PID. In these cases, we will perform functional studies to found a correlation between the genetic alteration and its relevance in the pathogenesis of immunological and clinical features, extended to larger cohort of similar patients.

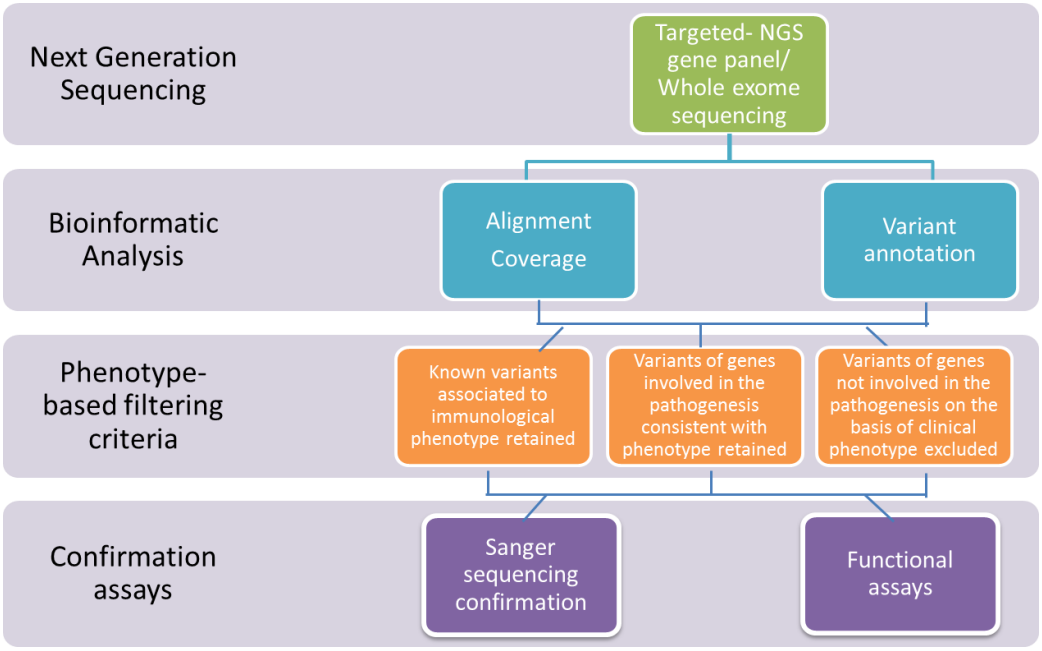
RESULTS

Patients

The patients who presented one or more of the following clinical criteria, associated to one or more immunological alteration, highly suggestive for primary immunological defect, were included into the study. Clinical criteria included: opportunistic infections; granuloma; CMC; intractable diarrhea; bronchiectasis; severe autoimmunity; variably associated to syndromic features and/or familiarity for similar phenotype. Immunological alterations included: abnormal lymphocyte subsets (absolute count < 2 SD of normal values according to ESID criteria); proliferative response to mitogens <10% of the CTR; absent/poor specific antibody

response; hypogammaglobulinemia; high IgE levels (>2000 KU/l); absent cytolytic activity; alteration of class switch recombination (CSR) with or without hyper-IgM. We identified fourty-five patients, heterogeneous for ethnic origin, age and sex who fulfilled the inclusion criteria. In 27 patients 571 genes, including genes involved in immunological pathways, were analyzed through NGS technology. In 18 WES was performed. After the detection of the variants, bioinformatics analysis of NGS results was performed through searches in variant databases, such as the Exome Variant Server (EVS, http://evs.gs.washington.edu/EVS). Subsequently, additional variant filtering criteria were established and optimized in order to streamline likely causative mutations (Figure 2). All the variants defined as potentially responsible for the clinical phenotype according to the phenotype-based filtering criteria, have been validated by classical Sanger sequencing and, subsequently, functionally tested in vitro.

Figure 2: Flowchart of filtering strategy



Genetic alterations

The alterations identified were divided into four groups, on the basis of NGS results, functional alterations and consistency between genotype, phenotype and immune functionality.

A fully satisfying molecular diagnosis of PID was achieved in 8 of 45 patients (17%).

The first group includes 4 subjects in which we identified a genetic defect associated to a well-known PID. In particular, mutationss in CD40L, BTK, STAT1 and JAK3 genes were identified and confirmed by Sanger sequencing (Table IV).

Table IV: Genetic variants associated to typical PIDs

Patients	Gene	Mutation	Protein	Zigosity	Inheritance	Clinical and immunological phenotype
001	*CD40L*	c.373C>T	p.His125Tyr	Hom	XL	Severe hypogammaglobulinemia with hyper IgM, neutropenia, P. carinii pneumonia, CMV infection, intractable diarrhea
002	*STAT1*	c.847T>A	p.Leu283Met	Het	AD	Chronic mucocutaneous candidiasis, recurrent pneumonia, hypothyroidism, lymphopenia, poor vaccine response
003	*BTK*	c.1105C>T	p.Leu369Phe	Hom	XL	Agammaglobulinemia
004	*JAK3*	c.856C>T	p.Gln286Ter	Hom	AR	T-B+NK- SCID, chronic diarrhea, pathological proliferative response to mytogens , IgA deficiency

The second group includes 4 subjects in which alterations of genes responsible for a well-known PID, were associated with atypical clinical features, which made very difficult the diagnosis through conventional strategies (Table V). In particular, in patient 005 we found a homozygous mutation in *MYD88* gene (c.192_194del; p.Glu66del). This patient was of Rom ethnia and resulted to have inherited the deletion from his unaffected consanguineous parents. Their familiar history was characterized by early infant deaths due to severe infections. The proband suffered from chronic yersiniosis, terminal ileitis, recurrent pyogenic adenitis without any inflammatory signs, high IgE levels (1190 IU/ml) and intermittent neutropenia. In reported cases, *MYD88* mutations are associated with susceptibility to invasive bacterial infections, particularly caused by gram-positive bacteria. Functionally, known mutations result in impairment of cytokine production after TLR stimulation. Patients 006 and 007 were found to carry homozygous deleterious mutations in PLDN and DOCK8/CLEC7A gene, respectively, whose phenotypic peculiarity has already been described in detail (41, 52). Briefly, patient 006 presented with partial oculocutaneous albinism, pyogenic dermatitis and impairment of NK cytotoxic activity that were associated to mutations of PLDN gene. In patient 007, who had chronic diarrhea, eczema, lymphopenia and elevated IgE levels, homozygous mutations of both DOCK8 and CLE7A genes were discovered (41, 52). In patient 008 we found a heterozygous (c.683C>T p.Pro228Leu) variant of unknown significance of CASP10 with exon 10 not covered at splice junction, associated with a heterozygous likely pathogenic variant of UNC13D (c.335G>C p.Cys112Ser). This patient had a clinical phenotype consistent with an autosomal dominant autoimmune

lymphoproliferative syndrome type II (ALPS-II) with the peculiarity of hypogammaglobulinemia and malignancies (LLA). Even though the CASP10 variant alone in the in silico prediction programs, Polyphen2 and Sorting Intolerant From Tolerant, was predicted to be tolerated and was observed in 35 healthy individuals (ExAc), its significance in association to a second deleterious variant has never been described and could be potentially relevant.

Table V: Genetic variants associated to novel presentation of PIDs

Patients	**Gene**	**Mutation**	**Protein**	**Zigosity**	**Inheritance**	**Clinical phenotype**
005	*MYD88*	c.192_194del	p.Glu66del	Hom	AR	Chronic yersiniosis and terminal ileitis, recurrent severe cutaneous granulomatous abscesses, hyper IgE, hypereosinophilia, neutropenia
006	*PLDN*	c.232C>T	p.Q78X	Hom	AR	Partial oculocutaneus albinism, nystagmus, recurrent cutaneous infections, thrombocytopenia, leucopenia, NK deficiency
007	*DOCK8/CLEC7A*	c.3193delA	p.Ser1065AlafsX17/p.Tyr238X	Hom/Hom	AR	Intractable diarrhea, eczema, malignancies, food allergies, hyper IgE, lymphopenia
008	*CASP10*	c.683C>T	p.Pro228Leu	Het	AD	Acute lymphoblastic leukemia treated with allogenic HSCT, ethmoiditis, recurrent lymphoadenopathy, autoimmune cytopenia,

	arthritis, hypogammaglobulinemia, hyperIgM, IgA deficiency

In the third group we included 4 patients, in which NGS revealed multiple genetic variants, that were consistent only with some features of the clinical phenotype (Table VI).

In the patient 009 we found a heterozygous mutation in CASP10 gene causative of the ALPS-II, characterized by autoimmune manifestations, particularly cytopenia, lymphoadenomegaly, splenomegaly and expansion of TCRαβ+ CD4/CD8 double negative T cells in the peripheral blood. His brother, with a similar phenotype, died at 1 year of age for haemophagocytic syndrome. The patient phenotype was characterized by spleen and liver enlargement, alopecia universalis, enamel tooth dysplasia, type 1 diabetes (T1D) and hyper-thyreotropinemia. In addition, the patient also had developmental delay, microcephaly, peculiar facial dysmorphism and skeletal abnormalities. His immunological phenotype was characterized by very high IgE levels (>2000 UI). The functional analysis of Fas-induced apoptosis in PHA-activated T cells from the patient confirmed that the apoptotic pathway was impaired, since cell survival upon triggering of Fas was 92% (normal values: median 60%, 95th percentile 82%).

Table VI: Genetic variants that are not causative of PID, with possible impact on the disease

Patients	Gene	Mutation	Protein	Zigosity	Major clinical features
009	*CASP10*	c.1202_1208del	p.Cys401LeufsTer15	Het	Alopecia universalis, hyperthyrotropinemia, type I diabetes mellitus, dental enamel hypoplasia, developmental delay, short stature, candidiasis, hepatomegaly, multiple skeletal abnormalities, myopia, dysmorphic features, microcephaly
010	*DOCK8* *TRL3*	c.1907A>G c.2672A>G	p.Lys636Arg p.His891Arg	Het	Inflammatory bowel disease, short stature, aspergillosis, EBV infection, low CD4+ lymphocyte subset , increased CD4 CD8 double negative T cells , normal antibody response
011	*ADA* *ERCC6*	c.377C>A c.1047A>G c.3262A>G c.2697G>A	p.Pro126Gln (reported in late onset SCID) p.K349 p.Ser1088Gly p.T899	Het	T-B+NK+ SCID treated with bone marrow transplantation, Pneumocystis carinii pneumonia, recurrent otitis, absent ossicular bone with hypoacusis of the right ear, mild brain and cerebellar atrophy, speech delay, idiopathic scoliosis
012	*AP3B1* *PRF* *ADAMTS13*	c.787G>T c.695G>A c.272C>T c.2701G>T	p.Gly263Cys p.Arg232His p.Ala91Val p.Ala901Ser	Het	Interstitial lung disease CMV infection,esophageal candidiasis, strabismus, abnormal expression of perforin in NK cells, reduction of CD4+ lymphocyte subset with increase of CD19+ and HLA-DR, normal proliferative response to mytogens, normal antibody response

013	*CFTR*	c.2991G >C	p.Leu997Phe	Het	Hypogammaglobulinemia late onset, recurrent pneumonia, bronchiectasis, chronic sinusitis, cervical and mediastinum lymphoadenopathy, recurrent abdominal pain, hepatomegaly with low grade steatosis, splenomegaly

The last group of patients includes patients with a complex disorder not classified in any known syndrome, associated with a number of genetic variants, each of them, on an individual basis, was not proved as relevant for the pathogenesis (Table VII). In this group, due to the limited number of cases observed, it was not possible to find out whether the array of the variations, on the whole, could be implicated in the pathogenesis. In these cases, we will perform functional studies to found a correlation between the genetic alterations and their relevance in the pathogenesis of immunological and clinical features, extended to larger cohorts of similar patients. Eventually, in the remaining 23 patient NGS, including 9 T-NGS and 14 WES, did not reveal any variant at all.

Patients	Gene	Mutation	Protein	Zigosity	Major clinical features
014	*SYCE2*	c.577G>A	p.Val193Met	Het	t(11;18)MLT1-AP12 gastric maltoma HP +, persistent oral candidiasis, sinusitis; lung cystis, chronic cough, recurrent fever, hypereosinophilia, reccurent itch, reccurent myofasciitis, hyper IgM, altered somatic hypermutation, absent CD19+ CD20- IgG+ (mature), low CD19+ CD27+ IgM+ (memory), absent CD19+ CD27+ IgM- (switched memory)
Sample 1089	*LYST*	c.10235G>A	p.Arg3412His		
015	*ATR*	c.5257A>G	p.Ile1753Val	Het	Severe aplastic anemia, hepatomegaly, Legionella p. and Aspergillus recurrent pneumonia, metacarpal deforming alterations with bone demineralization, abnormal lymphocyte proliferation, dilated cardiomyopathy, early retinophaty
Sample 1028	*ARSA*	c.869G>A	p.Arg290His	Het	
	CASP10	c.683C>T	p.Pro228Leu	Het	
	IKBIKG	c.1165C>T	p.Pro389Ser	Het	
	MEFV	c.460T>C	p.Ser154Pro	Het	
	SP110	c.1114C>T	p.Arg372Ter	Het	
	UNC13D	c.335G>C	p.Cys112Ser	Het	
016	*ATRX*	c.2247_2249del	p.Ser750del	Het	Autoimmune adrenal insufficiency, autoimmune thyroiditis, lymphadenopathy, autoimmune thrombocytopenia and neutropenia
Sample 1020		c.2133_2135del	p.Ser712del	Hom	
	MYD88	c.10_28del	p.Ala6ProfsTer39	Het	
	DOCK8	c.2920C>A	p.His974Asn	Het	
		c.3016C>A	p.His1006Asn	Het	
		c.3220C>A	p.His1074Asn	Het	
017	*TYK2*	c.3488A>G	p.Glu1163Gly	Het	Hypogammaglobulinemia, familial IgA deficiency, hyper IgE, multiple bronchiectasis, candidiasis
Sample 972					
018	*TLR3*	c.634-10C>A		Het	Familial IgA deficiency, multiple bronchiectasis, recurrent respiratory infections, low IgM levels
Sample 848					

019 **Sample 1051**	*CASP10*	c.1094A>C	p.Tyr365Ser	Het	Mild hypogammaglobulinemia, undetectable CD16+ lymphocyte levels, pervasive developmental disorder
	ERCC5			Het	
	GJC2			Het	
020 **Sample 1029**	*CD3-ZETA*	c.301C>T	p.Gln101Ter	Het	Hypogammaglobulinemia, recurrent pneumonia, previous alopecia, behavioral disorders, oropharyngeal candidiasis
	OCRL				

Table VIII: Genetic variants that are not causative of PID with undetermined impact on the disease

Discussion

The advent of NGS technologies, enabled physicians to investigate multiple genes in a single assay, providing great opportunities for diagnosing patients affected with complex disorders of the immune system with a heterogeneous genetic background. In this study, NGS panel with customized analysis for genes related to immunodeficiencies or immune functions was used to identify disease-causing mutations in patients affected with clinical phenotypes highly suggestive of an inheritable PID, still undiagnosed after using the traditional diagnostic procedures. Thanks to this novel diagnostic approach, a defined diagnosis of PID was achieved in a timely manner, in 8 out of 45 subjects.

Genetic alterations identified have been divided into 4 categories: I) genetic alterations associated to a canonical PID phenotype; II) novel or already described alterations of genes causing previously defined PIDs associated with novel clinical features; III) functionally relevant genetic alterations partially consistent with novel clinical phenotypes;

IV) multiple genetic variants not consistent with functional alterations individually, in whom the link between the genotype and that new phenotype is still missing.

In patients included in the first group, an extensive traditional diagnostic approach, according to worldwide accepted protocols (53), had been carried out but not allowed to reach a diagnosis in a timely manner. Giving the overlapping of clinical and laboratory among different PIDs, the possibility to sequence many different genes simultaneously, through NGS, may allow a rapid identification of the underlying molecular alteration (37). In the second category of patients, the molecular definition of the diagnosis greatly contributed to expanding the overall knowledge of pathogenetic mechanisms underlying that specific disorder. In these cases, the atypical presentation may lead to diagnostic delay. In the fourth group, no genotype-phenotype relationship was possible based on the current knowledge. The creation of a worldwide 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 same phenotypes and mutations in the same array of genes, would suggest that the sum of variations of different genes, is of some pathogenic significance, as either causative or modifier factor.

It should be considered that in the majority of the patients, NGS approach unfortunately resulted not suitable to detect neither novel nor known genetic alteration responsible for the clinical phenotype. Different aspects should be considered to interpret this finding: further genes in the NGS test panel could be required; in some cases, these genes may have not been proven as causative of a PID; in NGS technology only coding regions are amplified, while mutations could be located in intronic, promotor or regulator regions.

Moreover, all NGS techniques, due to the generation of short reads (54), show a low sensitivity to detect complex structural variations (deletions, insertions, inversions), repeat sequences or complex rearrangement. To overcome these technique limitations, WES or whole genome sequencing might be better strategies to deeply investigate these cases (40, 55) as a second line diagnostic tool.

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, which might allow clinicians to rapidly identify an affected gene, that, probably, would be never sought using the traditional approach based on a functional driven hypothesis. Prompt diagnosis may allow physicians to get started with the more appropriate treatment, which may often be life-saving.

CHAPTER 2

"STAT1 gain of function mutation in the pathogenesis of chronic mucocutaneous disease in the context of a complex multisystemic disorder"

CMC is a heterogeneous group of disorders characterized by noninvasive persistent Candida species infections of the skin, nails, and mucous membranes (56). Different immunological alterations may lead to susceptibility to Candida infections. Among secondary immunodeficiencies, infections with the human immunodeficiency virus and the prolonged use of glucocorticoids or antibiotics are able to predispose to fungal infections. CMC may also be an important feature of rare PIDs caused by alterations of genes implicated in cell mediated immunity necessary for fighting Candida infections (56-59).

Recent studies suggests the role of T helper 17 (Th17) cells and their effector cytokines interleukin 17 (IL-17) and interleukin 22 (IL-22), essential for mucocutaneous anti-fungal host defense (60-62) in the pathogenesis of CMC. Patients affected with the autosomal dominant form of hyper IgE syndrome (HIES) due to the deficiency of signal transducer and activator of transcription 3 (STAT3), which usually suffer from CMC (63-66), show a profound reduction of the numbers of IL-17 producing circulating T cells. Autosomal recessive mutations in the caspase recruitment domain-containing protein 9 (CARD9) also suffer from dermatophytosis and Candida meningitis (67). In autoimmune polyendocrinopathy candidiasis and ectodermal dystrophy (APECED)-syndrome CMC seems to be due to high titers of neutralizing autoantibodies against IL-17A, IL-17F and IL-

22 (68-70). Impaired IL-17 signaling observed in patients with IL17F, IL17RA or IL17RC mutations also lead to CMC (71, 72).

Gain-of-function (GOF) mutations in signal transducer and activator of transcription 1 (STAT1) have been recently identified through WES in a cohort of patients affected with CMCD (73). Heterozygous STAT1 GOF mutationsactually represent the most frequent underlying cause of CMCD patients (50 to 100% of the CMCD patients recruited, according to the various centers asked). Mutations have been identified in either the coiled-coil domain (CCD) or in DNA-binding domain (DBD) of STAT1. GOF mutations impair STAT1 nuclear dephosphorylation, in response to the stimulation with STAT1-dependent cytokines (IFN-α/b, IFN-γ, and IL-27), and STAT3-dependent (IL-6 and IL-21), leading to increased STAT1 phosphorylation (73-76). Alterations in the development of IL-17A- and/or IL-17F-producing T-cell lead to CMCD. Intracellular dimorphic fungal infections, disseminated coccidioidomycosis, and histoplasmosis have been also described in patients carrying STAT1 GOF mutations (77). Since 2011, a great number of patients with STAT1 GOF mutations have been described (73, 76-90). However, most reports have focused on the molecular and cellular defects of one or a small series of patients. This provides useful but incomplete clinical information. The comprehensive clinical features and outcomes of patients with STAT1 GOF mutations remain undefined.

2.1 Novel STAT1 gain of function mutation and suppurative infections

In this *Letter to the Editor* published on *Pediatric Allergy and Immunology*, we report on a 17-year-old patient with CMCC, recurrent herpetic infections and suppurative eyelid infections carrying a *de novo* heterozygous GOF mutation in exon 14 (p.T387A) of STAT1 in the DBD. This mutation has not been previously reported (91, 92). As previously described, the underlying pathogenic mechanism involves gain of STAT1 function due to impaired STAT1dephosphorylation. Consistently with previous reports, laboratory evaluation revealed persistently elevated IgE levels (684 KU/L), normal to low-normal lymphocyte cell counts, and reduced levels of switched memory B cells (89). STAT1 GOF mutations are considered responsible for very complex and variable phenotypes, characterized by susceptibility to herpetic (93) and fungal infections (77), autoimmunity, enteropathy, cardiac and vascular alterations, bronchiectasis (94), parodontitis and failure to thrive (75, 89).

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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 ($CD3^-$ $CD19^+$ $CD24^+$ CD38hiCD27$^-$; 8.2%),

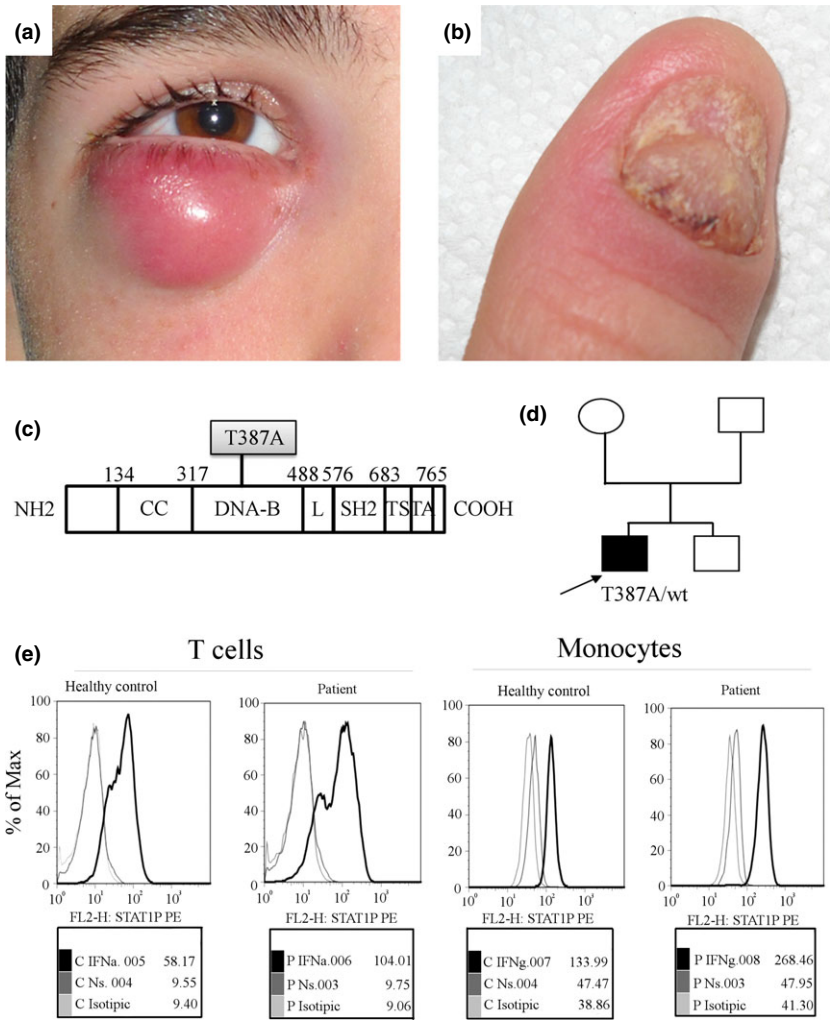

Figure 1 Clinical features. (a) Suppurative eyelid infection. (b) Onychomycosis. (c) Genomic sequence analysis of *STAT1* gene showing a T387A heterozygous mutation in the DNA-binding domain (DBD). (d) Family pedigree. The proband is indicated with an arrow. (e) Patient whole blood sample stimulated 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/loCD27$^-$; 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, differently from the control. The study of the T-cell compartment revealed normal representation of CD4 and CD8 naïve and memory T cells (CD4: 18.3% and 16.2%; CD8: 21.2% and 8.7%, respectively). We then evaluated the percentage of $CD4^+$ $IL\text{-}17A^+$ and $CD4^+$ $IFN\text{-}\gamma^+$ cells following PMA plus ionomycin stimulation for 6 h, to evaluate TH17 and TH1 development. The patient showed a lower number of $CD4^+$ $IL\text{-}17A^+$ cells than controls (0.25% vs. 1.66%) and increased percentage of $CD4^+$ $IFN\text{-}\gamma^+$ 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, recurrent herpetic infections, and suppurative eyelid infections carrying a *de novo* heterozygous GOF mutation in exon 14 (p.T387A) of STAT1 in the DBD. This mutation has not been previously reported (3, 4). As previously described, the underlying pathogenic mechanism involves STAT1 gain of function due to impaired STAT1 dephosphorylation. Consistently with previous reports, laboratory evaluation revealed persistently elevated IgE levels (684 kU/l), normal

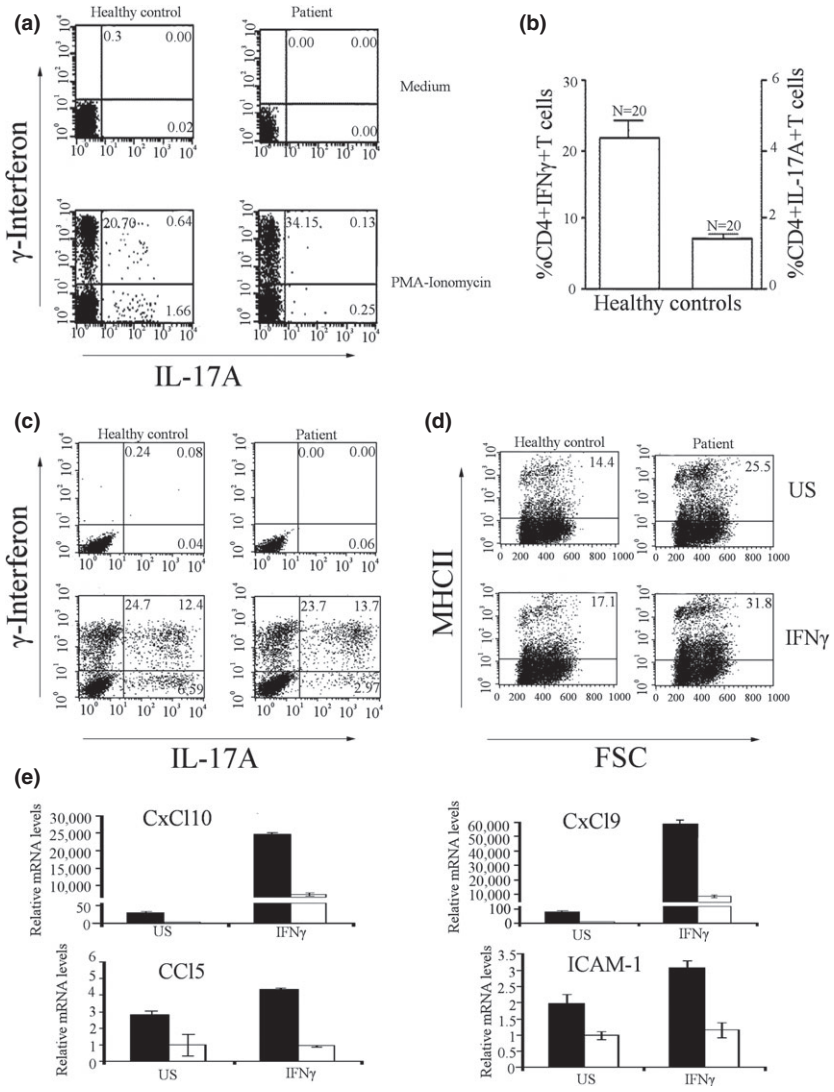

Figure 2 *STAT1* GOF mutation impairs TH17 development and increases the expression of STAT1-regulated genes. (a) Percentage of $CD4^+$ IL-$17A^+$ and $CD4^+$ IFN-γ^+ cells following PMA plus ionomycin stimulation for 6 h. The patient shows a lower number of $CD4^+$ IL-$17A^+$ cells than the control (0.25% vs. 1.66%) and increased percentage of $CD4^+$ IFN-γ^+ cells (34.15% vs. 20.70%). (b) Percentage of $CD4^+$ IFN-γ^+ and $CD4^+$ IL-$17A^+$ cells in 20 healthy controls (values expressed as mean ± SD). (c) $CD4^+$ IL-$17A^+$ and $CD4^+$ IFN-γ^+ 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-$17A^+$ development. $CD4^+$ IFN-γ^+ 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

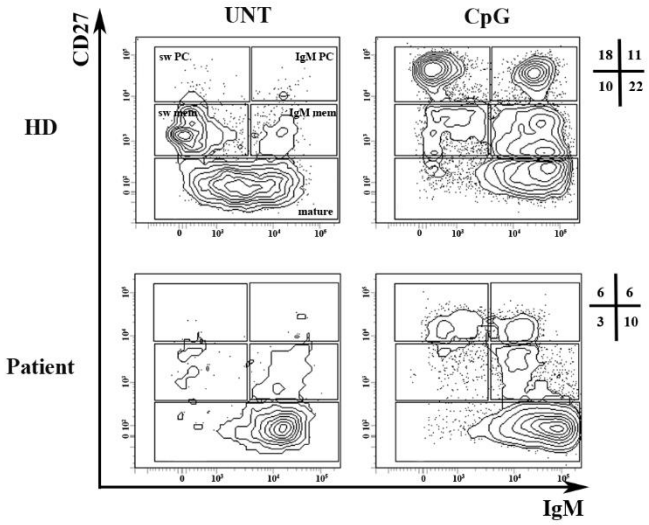
Additional Supporting Information may be found in the online version of this article:
Figure S1. PBMCs cultured with medium or CpG were stained with antibodies to CD27 and IgM at day 7.

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,

Supporting information

Figure S 1. 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 B cells express IgM and CD27, whereas switched memory lack IgM. Plasma cells are bright for CD27 and have or lack IgM. B cells from the patient carrying the STAT1 mutation adequately proliferated in response to CpG, and CD27 bright terminally differentiated plasma cells normally developed.



Conclusive remarks

In our patient, the clinical phenotype was dominated by recurrent foruncolosis, parodontitis and suppurative eyelid infections, mostly caused by Staphylococcus infections. Since 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.

2.2 Heterozygous STAT1 gain-of-function mutations underlie a broad clinical phenotype: an international survey of 234 patients from 140 kindreds

Julie Toubiana, Jean-Laurent Casanova, and Anne Puel in collaboration with working group on CMC

Introduction

STAT1 GOF mutations have been identified for the first time in 2011 in a cohort of patients affected with autosomal dominant CMC. Since their discovery a wide range of other clinical signs, including infectious and autoimmune diseases, cerebral aneurysms and carcinoma, has been described in patients from all over the world. Given the high variability of the clinical presentation and the fact that only small series of patients have been till now described, the comprehensive clinical features and outcomes of this syndrome remain, to date, undefined.

In this international survey, which has been submitted to *Blood* as *Full Paper*, Dr. J. Toubiana, in collaboration with the working group on CMC, described a large cohort of patients affected with CMC in order to better define the clinical features, the outcome, the

response to preventive and curative treatments, and immunological and hematological features of this complex syndrome.

RESULTS

Demographic features

A total of 234 patients from 140 kindreds originating from 35 countries on all the five continents were involved. The cohort was composed of a quite equal number of male and female with a M/F ratio 0.95. Median age of the patients at the time of the study was 23 years (range: 1 - 71 years).

Genetic features

A total of 65 different mutations in *STAT1* gene were identified. In the 63% there was a familial inheritance. In most cases (62%) the mutation involved the CCD, and in the 33% the DBD. The transactivation domain, the N-ter domain and the SH2 domain were affected in 2, 2 and 1% of the case, respectively.

Basic immunological phenotype

Immunological parameters including T cell proliferation to *Candida* were normal in most of the 199 analyzed patients (Table VIII). Low lymphocyte cell counts, or IgG/A levels were significantly associated with lower respiratory tract infections (LRIs), low proportions of $CD19^+$ or $CD4^+$ cell subsets with viral infections, and or low proportions of B and T-cell

subsets, hypogammaglobulinemia, and/or weak antibody (Ab) response with mycobacterial infections. Low levels of IL-17A-producing T cells were observed in 81% of the 32 patients tested.

Table VIII. Immunological investigations in patients with *STAT1* GOF mutation

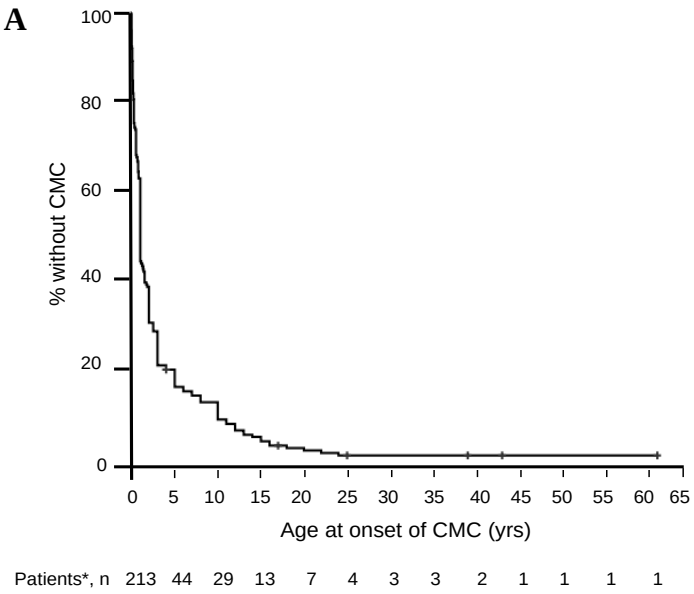
Biological investigations	**Patients tested (*n*)**	**Normal (%)**	**Low (%)**	**High (%)**
Total lymphocyte rate	199	83	17	-
$CD4^+$lymphocytes	184	73	27	-
$CD8^+$ T lymphocytes	176	81	17	2
$CD19^+$ or $CD20^+$ lymphocytes	175	81	19	-
$CD27^+CD19^+$ memory B lymphocytes	41	58	42	-
$CD16^+CD56^+$ lymphocytes	126	73	24	3
$CD3^+IL17A^+$ lymphocytes	32	19	81	-
T-cell proliferation (mitogen and/or antigen)	106	69	31	-
IgG levels (g/L)	195	76	4	20[1]
IgA levels (g/L)	183	82	14	4
IgM levels (g/L)	183	100	-	-
IgG1 levels (g/L)	83	100	-	-
IgG2 levels (g/L)	83	63	37	-

IgG3, 4 levels (g/L)	83	51	49	-
IgE levels (kIU/L)	119	96	-	4[2]
Antibodies against protein antigens[3]	88	78	22	-

[1]IgG>15 g/dL; [2]IgE>140 kIU/L: [3]tetanus, diphteria toxoid, or poliovirus

Fungal infections

As expected CMC was the most frequent clinical feature, observed in 97% of the patients. The remaining six patients, in which CMC was not identified, suffered from invasive fungal infection (1 patient), severe bacterial infections (5 patients), hypothyroidism (4 patients), and cerebral aneurysm (1 patient). The 26% of the patients developed different other superficial and invasive fungal diseases. The median age at the onset of CMC was one year (range: birth - 24 years) (Figure 3).



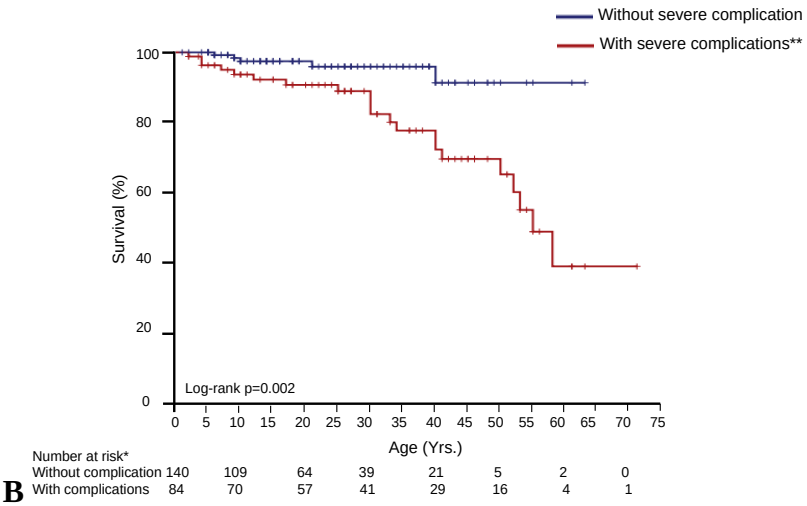


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Figure 3. Kaplan-Meier curves for onset of CMCD and outcome. (A) Onset of CMCD. * Age at onset of CMC was available for 213 patients. (B) Overall survival curves. * Age was available for 224 patients, ** patients with severe complications are patients who displayed invasive infections, aneurysms and/or tumors.

CMC affected the oral mucosae in the 94% of the cases (Table IX). Skin, esophageal/genital, and/or nail were involved in 61, 57, and 57% of the patients, respectively (Table IX). The culture of various specimens (skin, nails, throat, genital/esophageal mucosae) grew different *Candida* spp. (Table X). As expected, *C. albicans* was the species most frequent species, representing the 95% of the *Candida* isolates. Superficial dermatophytic infections of the scalp, skin or nails were suspected in 16% of the patients and were microbiologically confirmed (*Trichophyton* spp., *Microsporon* spp.) in 50% of these patients. The 10% of the patients experienced invasive fungal infections (Table IX). Invasive candidiasis was identified in 9 patients, and fungal pneumonia was observed in 14 patients (*Cryptococcus*

spp. (*n*=2), *Pneumocystis jiroveci* (*n*=5), *Aspergillus* spp. (*n*=5), and *Histoplasma* spp. (*n*=2)). Two patients had fungal nephritis (*C. curvatus* and *Trichosporon asahii*), two patients suffered from cryptococcal meningitis, and two patients displayed disseminated fungal disease (*Coccidioides* spp. or Mucoraceae) (Table X).

Bacterial infections

Bacterial infections were observed in the 74% of the patients studied (Table VIII). LRIs, including recurrent lobar pneumonia, recurrent bronchitis, and/or interstitial pneumonia were reported in the 64% of the patients (Table IX). The most frequent causative pathogens were *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *S. aureus* were (Table X). Recurrent or chronic, sinusitis or otitis media were observed in the 42% of the patients. Other bacterial infections included tonsillitis and recurrent conjunctivitis/keratitis observed in 13% of the patients, and blepharitis or ectropion in the 2% of the patients. Recurrent skin infections were found in the 36% of the patients (Table IX). Skin infections mostly included folliculitis, followed by cellulitis, abscesses, and paronychia. *S. aureus* was isolated in most cases. Severe gastroenteritis, sepsis, or bone and joint infections have also been described. Mycobacterial diseases, including lung and skin disease and adenitis caused by tuberculosis and non-tupercolosis Mycobacteria, developed in the 6% of the patients.

Viral infections

Severe or atypical viral infection, including recurrent mucocutaneous viral infections, severe chickenpox, history of shingles during childhood, *Molluscum contagiosum* or warts, and severe systemic infections were observed in 38% of the patients (Table IX). The main causal agent was herpes simplex virus (HSV-1 or HSV-2; Table 3). Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) were the viruses most frequently found in severe systemic infections (Table X). Five patients had CMV disease with proven organ infection, including one patient with retinitis, two with ulcerative digestive infections, and two with lung infections, one of whom also had encephalitis. Chronic active EBV infections were also observed and were less severe, not requiring any specific treatment. In few cases severe HHV-6 and parvovirus infection leading to severe sepsis or hemophagocytosis were described. In one BK-virus urinary tract infection with functional consequences was obsetved. Severe disease due to live virus vaccines was observed in 2 patients displayed (small-pox and measles). Two patients had chronic hepatitis C infection leading to cirrhosis.

Table IX. Sites of infection in patients with *STAT1* GOF mutations

Type of infections **Patients, (%)**	**Total** ***n*=234**
Mucocutaneous fungal infections	**228 (97)**
Thrush	214 (94)
Cutaneous mycosis	137 (61)
Onychomycosis	131 (57)
Esophageal / Genital mycosis	129 (57)

Aphtous stomatitis	100 (44)
Scalp mycosis	49 (21)
Invasive fungal infections	**24 (10)**
Invasive candidiasis	9 (41)
Other invasive infections	20 (83)
Bacterial infections[1]	**174 (74)**
LRI	110 (64)
ENT	99 (59)
Skin	63 (36)
Other[2]	18 (10)
Mycobacterial infections	**15 (6)**
Lung disease	5 (33)
Adenitis / skin disease	6 (40)
Disseminated disease	4 (27)
Viral infections[1]	**90 (38)**
Cutaneous	78 (87)
Systemic	20 (22)

LRI: Lower respiratory tract infection; ENT: ear, nose and throat. [1]Probable or proven bacterial / viral infection. [2]Severe acute gastroenteritis, septicemia, bone and joint infections

Table X. Documented infections associated with STAT1 GOF

Associated pathogens	**Documented infections (%)**
Mucocutaneous fungal infections	***n*=149**
Candida albicans	121 (81)
Dermatophytes spp.	19 (13)
Other[1]	9 (6)

Invasive fungal infections	***n*=29**
Candida spp.	9 (31)
Pneumocystis jiroveci	5 (17.2)
Aspergillus spp.	5 (17.2)
Cryptococcus spp.	5 (17.2)
Histoplasma spp.	2 (7)
Trichosporon spp.	1 (3·5)
Coccodioides spp.	1 (3·5)
Apophysomyces spp.	1(3·5)
Bacterial infections	**n=72**
S. aureus	19 (26)
Streptococcus spp.	16 (22)
Enterobacteriaceae	12 (17)
Pseudomonas aeruginosa	9 (12.5)
Haemophilus influenza	7 (10)
Other[2]	9 (12·5)
Mycobacterial infections	***n*=15**
M. tuberculosis	4 (26·6)
M. bovis or BCG strain	4 (26·6)
M. avium	3 (20)
Other[3]	4 (26·6)
Viral infections[4]	***n*=138**
Herpes simplex	42 (31)
Varicella-Zoster	40 (29)
Molluscum contagiosum /Warts	25 (18)
Cytomegalovirus	12 (8·5)

Epstein Barr virus	10 (7)
Other[5]	9 (6·5)
Parasitic infections[6]	***n=2***

[1]*C. glabrata, C. tropicalis, C. kefir, C. membranaefacie, C. famata* and *Malassezia furfur;*

[2]*Campylobacter* spp., *Stenotrophomonas maltophilia*, anaerobic pathogens, *Neisseria meningitides*; [3]*M. fortuitum, M. mucogenicum* or not identified; [4]Clinically probable or microbiologically confirmed; [5]*HHV6, Parvovirus, BK* virus, *hepatitis C*, live virus vaccine disease; [6]Giardiasis, visceral leishmaniasis

Autoimmune and inflammatory diseases

In the 44% of the patients, clinical autoimmunity and/or laboratory autoimmune markers were documented (Table XI). Median age at the onset of the autoimmune manifestations was 24 years (range: 3·5 years -71 years), with a slight prevalence in females (M/F sex ratio 0·74). The 39% of the patients displayed clinical autoimmune manifestations, and 17% of these patients had more than one clinical autoimmune disorder. The most frequent autoimmune manifestation was autoimmune thyroid disease with hypothyroidism, requiring hormone substitution. Other autoimmune manifestations included T1D, vitiligo, alopecia, or psoriasis, systemic lupus erythematosus (SLE), scleroderma, pernicious anemia , celiac disease, autoimmune, hematologic autoimmunity (chronic hemolytic anemia or autoimmune thrombocytopenia during childhood), ankylosing spondylitis, multiple sclerosis, inflammatory bowel disease (Crohn's disease, enteropathy with lymphocytic infiltration, and ulcerative colitis). Auto-Abs were found in the 43% of the patients. Auto-Abs were detected in the absence of clinical symptoms in 21% of the patients tested, mostly anti-nuclear Abs (87%) and anti-thyroid antibodies (13%).

Table XI. Other clinical features and outcomes of patients with *STAT1* GOF mutations

Non-infectious phenotypes **Patients, (%)**	**Total** ***n*=234**
Autoimmunity/inflammatory disease	**92 (39)**
Hypothyroidism	54 (59)
Other endocrine disease[1]	14 (15)
Skin disease[2]	25 (27)
Gastrointestinal disease[3]	9 (10)
Autoimmune hepatitis	6 (7)
Autoimmune cytopenia[4]	8 (9)
Other[5]	3 (3)
Aneurysm	**17 (7)**
Cerebral	14 (82)
Extracerebral	3 (19)
Tumor	**13 (6)**
Benign	2 (15)
Squamous cell carcinoma	8 (62)
Gastrointestinal carcinoma	2 (15)
Other[6]	2 (15)
Other clinical features	
Asthma / eczema	40 (17)
Bone fragility	5 (2)
Clinical outcome	
Death	27 (12)
Dysphagia/ esophageal stenosis	22 (9)
Failure to thrive	17 (7)
Bronchiectasis	48 (21)

[1]Diabetes mellitus, Addison's disease, GH deficiency; [2]SLE, vitiligo, psoriasis, alopecia, scleroderma; [3]Biermer anemia, celiac disease, colitis; [4]Immunologic anemia or thrombocytopenia; [5]Multiple sclerosis, ankylosing spondylitis; [6]Melanoma, basal cell carcinoma

Other clinical features

Patients included in this study displayed an unexpected high number of aneurysms 7% (Table XI) with a median ageat the diagnosis of 23 years (range: 3-50 years). The rate was higher as compared to the standard population (~3%), with no difference between the sexes. Aneurysms were more frequent in patients with underlying autoimmunity, and in particular with endocrine (27% vs. 6%, $p<0{\cdot}01$). Diagnosis was based on symptoms and radiologically confirmed in 15 patients. Systematic radiologic investigations identified two additional patients. Cerebral imaging was also carried out for 20 other asymptomatic patients and yielded normal results. Symptoms included hemorrhages in 50% of the patients, and abdominal pain, and neurological signs, such as hemiplegia, seizures or attention lapses in the remaining. In most cases aneurysms were multiple and located in the cerebral vascular system (82%) (basilar trunk, vertebral arteries, and cerebral and intracranial carotid arteries). Extracerebral aneurysms were found in the abdominal aorta, iliac arteries, and lung arteries. Histological and microbiological investigations performed in one patient were (P19) negative. Other neurological signs included cerebral vasculitis, epilepsy, polyneuropathy, and cognitive disability. Carcinomas of the skin, gastrointestinal tract or larynx were observed in the 10% of the patients (adjusted to the 2000 US Standard population) with a slight predominance of female patients (56%, Table X). The frequency of cancer was higher than those observed in the standard population (~3%) (95). Tumors were more frequent in patients with a history of esophagitis (10% vs. 2%, $p<0{\cdot}01$). Asthma and eczema, and signs of allergy were observed in the 17% of the patients, with a frequency similar to the general

population and without unusual severity (96). Failure to thrive was observed in 7% of the patients.

Preventive and curative treatment

Most of the patients (72%) patients needed systemic long-term antifungal treatment (Table XII). Fluconazole was the major agent used for first-line treatment, followed by itraconazole or posaconazole. The 41% of the patients displayed clinical resistance to at least one antifungal, and the 11% of patients showed resistances to more than one antifungal. Most of these patients required second- or third-line treatments, such as voriconazole, echinocandins, terbinafine or liposomal amphotericin B. Clinical resistance to antifungals was related to a more severe phenotype (recurrent LRIs: 62% vs. 47% p=0·04, systemic fungal infections: 16% vs 7% p=0·03, mortality: 20% vs. 5%, p=0·001). The 24% of the patients with recurrent LRI or cutaneous staphylococcal disease also received antibacterial prophylaxis; cotrimoxazole was the mainly used agent. The 14% of the patients received polyvalent IgG infusions. GM-CSF infusions, interferon alpha, and then acitretin, did not improved CMC or herpes virus infections. Only in one patient G-CSF, lead to a marked improvement in CMC lesions. Three patients underwent allogeneic hematopoietic stem cell transplantation (HSCT) because of severe CMCD and recurrent bacterial infections; two of them died several months after HSCT, one from disseminated CMV at 30 years (P135, HLA- identical HSCT), and the other from interstitial lung disease at 2 years (P165, HLA-identical sibling HSCT). The last one is currently alive without serious complications (P136, HLA-identical cord blood transplantation). Five patients underwent immunosuppressive treatment (cyclosporine,

aziathoprine, corticosteroids or mycophenolate mofetil) for severe autoimmune disorders, and had a good clinical response, without serious infectious complications.

Table XII. Treatments of patients with STAT1 GOF

Treatment	**Patients, (%) *n*=234**
No antifungal treatment	**6 (3)**
Intermittent antifungal treatment	**45 (20)**
Current long-term antifungal treatment	**168 (72)**
Local treatment only	7 (4)
Oral fluconazole	112 (66)
Oral posaconazole / itraconazole	49 (29)
Oral voriconazole	13 (8)
I.V. echinocandins	5 (3)
Oral terbinafine	3 (2)
I.V. amphotericin B	3 (2)
Antibiotic prophylaxis	**57 (26)**
Cotrimoxazole	38 (67)
Macrolides	15 (26)
Other*	10 (18)
Antiviral prophylaxis	**4 (2)**
Polyvalent immunoglobulins	**30 (13)**
Immunotherapy**	**5 (2)**
Immunosuppressive therapies***	**5 (2)**
Hematopoietic stem cell transplantation (HSCT)	**2 (1)**

* nebulized colimycin, topical fucidic acid, tetracycline, amoxicillin

** GCSF/GM-CSF, interferon alpha/gamma

*** cyclosporine, aziathoprine, corticoids or mycophenolate mofetil

Clinical outcome

Secondary gastrointestinal complications, such as severe dysphagia or esophageal stenosis developed in the 10% of the patients and in in most cases they were associated with a history of recurrent esophageal candidiasis. Pulmonary complications including bronchiectasis and cystic pulmonary lesions developed in the 21% of the patients. All the patients had a previous history of recurrent pneumonia or bronchitis (Table XI), and displayed acute secondary infectious episodes caused by *P. aeruginosa*, *S. aureus*, non-tuberculous mycobacteria or *Enterobacteriaceae*. One patient also had an associated pneumatocyst, and three patients underwent lobectomy.

The 12% of the patients died (Table X) at a median age of 30 years (range: 2-58 years). The main causes of death were severe infections (including disseminated BCG disease, histoplasmosis, coccidioidomycosis, CMV, or bacterial LRI), cancer, and cerebral hemorrhage due to aneurysm. These three complications were the strongest predictors of poor outcome (25% mortality, versus 4% otherwise) (Figure 3). Also low total lymphocyte count was associated with a higher mortality rate (16% vs. 5%, p=0·008). The other patients died from fulminant hepatitis (1 patient from an unknown cause, and 1 from autoimmune hepatitis), complication of HSCT (in two patients) and unrelated causes (in four patients).

Discussion

This study confirmed that CMC is the most common infectious manifestation in patients carrying *STAT1* GOF mutations, with a very high penetrance, (only six *STAT1* GOF patients never had CMC at a median age of 31 years). In many cases Candida may be resistant to

azole treatments. This study also showed that these patients may often suffer from viral, bacterial, and other fungal infections. Autoimmunity, as well as aneurysms and carcinomas, were much more frequent in this cohort of patients than the general population. The presence of these complications accounts for a poor outcome. CMC typically begins in the first year of life. However, it may also appear for the first time up to the third decade of life. Clinical features are very variable within and between families (73). CMC probably develop as consequence of impaired IL-17A and IL-17F immunity (58, 71-73, 75, 81, 86, 91, 92, 97). Fungal infections also included superficial dermatophytosis (98), and invasive infections by a variety of yeasts and moulds (75, 77, 85, 87). Bacterial infections included cutaneous and bronchopulmonary infections caused by *S. aureus*. The occurrence of staphylococcal skin suggests that impaired STAT3-dependent IL-6 signaling may also be involved (73, 99). Low IgA, IgG2, and IgG4 levels as well as a poor Ab response, may favor respiratory tract infections as documented in some patients (100, 101). Viral disease might be due to exhaustion of virus-specific T cells (102), and mycobacterial disease to refractory responses to IFN-γ (77).

Autoimmunity, aneurysms, and tumors also features this syndrome. Autoimmunity is not observed in patients with inborn errors of IL-17A/F immunity or in patients with AD HIES. The enhanced autoimmunity observed in such patients likely result from stronger IFN-α/β signaling, as some of these autoimmune signs are observed in patients treated with IFN-α (e.g. thyroiditis) and in patients with type I interferonopathies (e.g. SLE) (103). The outcome of patients with *STAT1* GOF mutations is poor. Premature death may results from infections (33%), aneurysms (19%), cancer (19%), or fulminant hepatitis (7%). Infections

represent the major cause of premature death in these patients (59, 104). The proportion of patients with cerebral aneurysm has probably been underestimated, since radiological investigations were performed for only 37 patients, including in 15 patients with clinical signs suggestive of aneurysm. Given the high morbidity and mortality of cerebral aneurysms, systematic radiological screening would be warranted in all patients. The pathogenesis of aneurysms may due to conjunctive tissue abnormalities, as in patients with STAT3 deficiency (104), autoimmune, as in cerebral vasculitis, or infectious, as in systemic *Candida* infection (59). In this cohort, patients with aneurysms did not differ markedly from the others in terms of invasive fungal infection rates (p=0·6), but they seemed to display more endocrine autoimmune disorders (p=0·02). The frequency of skin and ENT carcinomas was high. These conditions probably result, at least in part, from CMC and the muco-cutaneous inflammation (especially esophagitis) (105). Patients with esophagitis and dysphagia should be regularely screened for tumor by sequential biopsies of the esophagus. Taken together, *STAT1* GOF mutation-associated AD CMCD should not be considered benign and should be handled at centers with experience in the diagnosis and management of such patients.

The heterogeneity of clinical care for the patients in this cohort makes it difficult to issue uniform recommendations concerning optimal management. The high rate of CMC resistance to antifungal treatments is a major issue. IgG infusion should be considered for patients with recurrent LRIs, with or without detectable signs of Ab deficiency. GM-CSF and G-CSF treatments have been proposed as a way of enhancing IL-17 T-cell differentiation (106-108). However, despite an encouraging recent report (83), these adjuvant therapies were useful in only one of the five patients in which they were tried. IFN-α/β blocking

antibodies, may alleviate autoimmune features. Treatments targeting the JAK-STAT pathway, such as the JAK1/2 inhibitor ruxolitinib, which has been approved for myelofibrosis treatment, or inhibitors of STAT1 activity for specific use in patients with GOF *STAT1* mutations, have shown significant clinical efficiency and might become the treatment of choice (76). The options must be weighed up carefully, bearing in mind the various anti-infectious, anti-tumor, and autoimmune effects of each cytokine. HSCT does not appear to be a viable option at the present time. It was performed in three patients with severe and recurrent fungal and viral infections, but two of them died. In conclusion, *STAT1* GOF mutations are the most common known genetic etiology of CMCD and are found in about half the patients studied (59, 109), and is associated with significant clinical pathology. Disease severity results from the deleterious impact of CMCD on quality of life, and the poor outcome associated with infections, autoimmunity, aneurysm, and carcinoma.

2.3 Clinical heterogeneity of dominant chronic mucocutaneous candidiasis disease: presenting as treatment-resistant candidiasis and chronic lung disease

In this *Article* published on *Clinical Immunology,* we report on nine patients carrying already known or novel GOF-STAT1 mutations. Three novel mutations were identified in the CCD, and in the DBD. As expected, CMCD represents the main manifestation and feature of suspicion at onset, but its severity largely varies from chronic recurrent and debilitating infection to mild forms, detectable after detailed collection of medical history, or occurring

only during antibiotic therapy. In some patient, a high risk of developing resistance to oral antifungal treatment, associated to high MIC to azoles (i.e. fluconazole), was observed and resulted in chronic infection with Candida. Development of antifungal resistance was not associated to any specific STAT1 mutations. Intermittent regimens of antifungal treatment or use of azoles at suboptimal doses might favor the selection of azole-resistant strains of Candida. For this reason, we suggest that antifungal prophylaxis with fluconazole or itraconazole should be started in all patients at the time of diagnosis, regularly continued and strictly monitored by pharmacokinetic measurements and susceptibility tests, in order to optimize control of diffuse candidiasis, prevent the development of inadequate dose-related resistance, and guarantee efficient treatment. Development of azole-resistance may require use of alternative regimens including caspofungin, or liposomal amphotericin B, which are administered by an intravenous route. As observed in the international survey patients may suffer from a broad spectrum of infection including recurrent bacterial infections since early childhood, and viral diseases mainly with herpes family viruses (88, 90, 93, 94). These infections may cause high morbidity and may increase the mortality risk. Lymphopenia, or autoimmunity may also complicate the prognosis. In our cohort, lymphozpenia (reduction in CD3+, CD4+ and CD8+ lymphocytes subsets) was not always present, but might progress by adulthood. Thus, a careful monitoring of the main lymphocyte subsets could help in clinical management by early detection of patients developing severe lymphopenia that might require to be evaluated for hematopoietic stem cell transplantation (HSCT). Lung disease represented a major complication as it may slowly evolve in adulthood to permanent lung damage predisposing to recurrent pulmonary exacerbations and higher morbidity. Early

recognition of lung damage at initial stage associated to prompt aggressive treatment of pulmonary infection and daily respiratory physiotherapy might prevent progression of the disease. The role of antibiotic prophylaxis, could be controversial because of increased risk of progressing candidiasis. Autoimmune manifestations represent common features of the disorder and extensive autoimmune assessment should be regularly performed: consistently with what previously reported, thyroidopathy represents the most common disorder (73, 91).

Conclusive remarks

Similarly to what observed in the international survey, in this small cohort of Italian patients we observed a great heterogeneity of age distribution and phenotypes.

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Clinical heterogeneity of dominant chronic mucocutaneous candidiasis disease: presenting as treatment-resistant candidiasis and chronic lung disease

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ABSTRACT

In gain-of-function *STAT1* mutations, chronic mucocutaneous candidiasis disease (CMCD) represents the phenotypic manifestation of a complex immunodeficiency characterized by clinical and immunological heterogeneity. We aimed to study clinical manifestations, long-term complications, molecular basis, and immune profile of patients with dominant CMCD. We identified nine patients with heterozygous mutations in *STAT1*, including novel amino acid substitutions (L283M, L351F, L400V). High risk of azole-resistance was observed, particularly when intermittent regimens of antifungal treatment or use of suboptimal dosage occurs. We report a case of Cryptococcosis and various bacterial and viral infections. Risk of developing bronchiectasis in early childhood or gradually evolving to chronic lung disease in adolescent or adult ages emerges. Lymphopenia is variable, likely progressing by adulthood. We conclude that continuous antifungal prophylaxis associated to drug monitoring might prevent resistance to treatment; prompt diagnosis and therapy of lung disease might control long-term progression; careful monitoring of lymphopenia-related infections might improve prognosis.

1. Introduction

In 2011, autosomal dominant gain-of-function (GOF) mutations in signal transducer and activator of transcription 1 (*STAT1*) gene have been identified as a cause of chronic mucocutaneous candidiasis disease (CMCD) [1,2]. To date, more than 100 patients have been reported, with 37 different mutations affecting the coiled–coiled (CCD) or the DNA-binding (DBD) domains of *STAT1*. This transcriptional activator plays a major role in various signaling pathways, particularly of IFNs, IL-27, IL-21, IL-6, and IL-17 immunity [3,4]. *STAT1* loss-of-function (LOF) mutations were already known to account for rare autosomal recessive (AR) immunodeficiencies [5], resulting respectively in fatal mycobacterial and viral diseases (when complete gene deficiency occurs [6]), or milder phenotypes (when deficiency is partial [7]). Autosomal dominant (AD) LOF mutations were identified as a cause of Mendelian susceptibility to mycobacteria infections [8]. GOF-STAT1 mutations were firstly described among patients affected with severe

Abbreviations: GOF, Gain-of-function; STAT1, Signal transducer and activator of transcription 1; CMCD, Chronic mucocutaneous candidiasis disease; CCD, Coiled–coiled domain; DBD, DNA-binding domain; LOF, Loss-of-function; AR, Autosomal recessive; AD, Autosomal dominant; CMC, Chronic mucocutaneous candidiasis; IPEX, Immune dysregulation, polyendocrinopathy, enteropathy, X-linked; WES, Whole exome sequencing; IFN, Interferon; pSTAT1, phosphorylated signal transducer and activator of transcription 1; ISRE(s), Interferon-stimulated response element(s); AIRE, Autoimmune regulator; PBMCs, Peripheral blood mononuclear Cells; EBV, Epstein–Barr virus; GAS, γ-Activated Sequence; MIC, Minimal inhibitory concentration; TSH, Tyrhoid-stimulating hormone; SLE, Systemic lupus erythematosus; TRECS, T-cell receptor excision circles; CT, Computed-tomography; PEP, Positive expiratory pressure; FEV1, Forced expiratory volume in 1 s; FEF 25–75%, Interval 25–75% of the forced vital capacity; G-CSF, Granulocyte-colony stimulating factor; GM-CSF, Granulocyte monocyte-colony stimulating factor; HSCT, Hematopoietic stem cell transplantation; MFI, Mean intensity fluorescence; HD, Healthy donor.

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CMCD of the skin, nails, and mucous membranes, associated to autoimmune diseases; life-threatening conditions such as squamous-cell carcinoma, cerebral and, recently, aortic aneurysms [9] were also rarely reported. Disseminated fungal (coccidioidomycosis, histoplasmosis, mucormycosis) and viral (herpes virus family, a case report of orf virus) infections were also described [10–13]. Mutations of *STAT1* have been identified by next generation sequencing also in patients with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked)-like features [14], and in patients with severe, fatal phenotypes resembling combined immunodeficiency [10,15]. Any evidence of any genotype–phenotype correlation is emerging, and data about long-term morbidity and prognosis may be controversial. Herein, we report nine patients diagnosed with previously reported and novel GOF-*STAT1* mutations, showing large heterogeneity for age distribution and phenotypes.

2. Material and methods

2.1. Patients

We report nine patients who were diagnosed as affected with CMCD and evaluated for genetic analysis and *in vitro* functional studies from their peripheral blood, according to approved protocols of Spedali Civili in Brescia, Italy. Cases are described in details in [16]. Written informed consent was obtained from all patients or their parents (for minors), and for healthy controls. Medical history and clinical data were retrospectively collected from medical records. In all patients extended immunological assessment was performed. For patients under the care of the Department of Brescia a pulmonary evaluation, including assessment of pulmonary function tests and imaging, was carried out.

2.2. Molecular genetic analysis

Whole exome sequencing (WES) was performed at Children's Mercy Hospital and Clinics, Kansas City, Centre for Pediatric Genomic Medicine (Professor Kingsmore Stephen F) for P1. *STAT1* mutation was subsequently confirmed by Sanger sequencing in both P1 and his mother P2. In patients P3–P9 Sanger sequencing was used to analyze *STAT1*. DNA was isolated from whole blood using QIAamp DNA Blood Mini Kit (Qiagen). *STAT1* gene was amplified by PCR and products were sequenced using BigDye Terminator Kit (Applied Biosystems). Sequences were analyzed with 310 Genetic Analyzer (Applied Biosystems). Sequence variants were identified relative to a reference sequence, GenBank accession no. ENST00000361099 for the *STAT1* cDNA, in which the c.1 position corresponds to the A of the ATG translation initiation codon. Mutations are designated as recommended by den Dunnen and Antonarakis [17].

2.3. Analysis of phosphorylated STAT1 (pSTAT1) by flow cytometry

Peripheral blood was left unstimulated and stimulated with IFNγ (1000 U/ml), or IFNα (40,000 U/ml), for 30 min. Cells were lysed, permeabilized, and stained, as indicated by the manufacturer (BD Phosflow). Specific phycoerythrin-labeled antibody for phosphorylated STAT1 (pSTAT1) (pY701; BD Biosciences) was used. pSTAT1 was evaluated in both lymphocyte and monocyte gates. Cells were acquired using FACSCalibur (BD Bioscence) and analyzed by FlowJo version 7.5 Software (TreeStar).

2.4. Cytoplasmic and nuclear extracts preparation, Western blot and EMSA

After stimulation with IFNγ or IFNβ for 30 min, cells were lysed in cold buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.4, 1% NP40, and protease inhibitors -Roche-containing 0.2 μg of aprotinin, leupeptin and 1 mmol/L of sodium orthovanadate) on ice for 15 min. For Western blot analysis, cytoplasmic extracts were resolved on 8% polyacrylamide and subjected to immunoblots by standard procedures. Nitrocellulose membranes were first blocked for 1 h at room temperature in TBST containing 5% BSA, and then incubated overnight at 4 °C with specific primary Abs (pSTAT1, STAT1) in the same buffer. Antibodies against phospho-tyrosine STAT1 were purchased from Cell Signaling Technologies (Denver, MA, USA). Antibodies against total STAT1 (sc-346) were obtained from Santa Cruz, and antibodies against β-actin were obtained from Sigma-Aldrich. Detection was carried out using HRP-conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences), and revealed using ECL system (Amersham Biosciences). For EMSA, after stimulation with IFNγ and/or IFNβ for 30 min, EBV transformed B cells (5×10^6/condition) were diluted in ice-cold PBS and centrifuged twice at 300 X g for 5 min at 4 °C. Nuclear extracts were prepared using a modification of the method of Dignam *et al.* [18]. Transcription factor-binding analyses were performed as described previously [18]. Nuclear extracts were incubated in binding buffer in the presence of the labeled oligonucleotide STAT-binding probe from the GRR element located within the promoter of the FcγRI/CD64 gene (5-CTTTTCTGGGAAATACATCTCAAATCCTTGAA ACATGCT-3) or from the interferon-stimulated response element (ISRE) (5-GATCGGGAAAGGGAAACCGAAACTGAA-3).

2.5. Mutagenesis assay

eGFP STAT1 WT vector (Addgene) was used to obtain mutated STAT1 form carrying L351F and L400V variants. Mutations were generated by site direct mutagenesis QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies).

2.6. Luciferase reporter assay

U3C cells were seminated into 96-well plates (1×10^4/well) and transfected with 100 ng/well reporter plasmids and plasmids carrying alleles of STAT1 (L351F and L400V) or a mock vector with Arrest-in (Thermo Scientific). After 6 h, cells were transferred into medium containing 10% FBS and cultured for 24h. Cells were stimulated with IFNγ at different concentration (10, 100, 1000 UI/ml), and IL-27 (100 ng/ml) for 16h. Luciferase production was assesed with Dual-Glo luciferase assay system (Promega) and normalized with respect to Renilla luciferase activity. Data are expressed as fold inductions with respect to unstimulated cells.

2.7. Statistical analysis

Statistical significance of *in vitro* immunological studies was analyzed by nonparametric two-side Mann–Whitney U-test with 95% confidence bounds. For all analyses $p < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. Genetic findings

We identified a novel familial *STAT1* mutation (L283M) in P1 (by WES) and in his mother P2. Other two previously unreported sporadic mutations were detected respectively in P3 and P9 (L351F), and in P6 (L400V). P3 had been previously analyzed for *AIRE* (autoimmune regulator) gene, showing a heterozygous variation 769C > T (R257X) he inherited from his healthy mother. Other mutations were: T385M in P4 and P5 [19], A267V in P7 [2], and T387A in P8 (Pignata personal communication; Higgins *et al.* [20]). All are heterozygous missense mutations (Table 1). Novel *STAT1* mutations affect both CCD and DBD domains.

3.2. Novel mutations result in increasing STAT1 phosphorylation

We investigated IFNα- and IFNγ-induced pSTAT1 by cytometry from peripheral blood mononuclear cells (PBMCs) of our patients compared to healthy controls (Fig. 1 in [16]). Overall, we observed

Table 1
STAT1 mutations in CMCD patients.

	Sex	Age (years)	Mutation (c.DNA)	Mutation (aa change)	Exon	Affected domain
P1	Male	11	c.(847 T > A)	L283M	Exon 10	CCD
P2	Female	45	c.(847 T > A)	L283M	Exon 10	CCD
P3	Male	33	c.(1441 G > T)	L351F	Exon 12	DBD
P4	Male	15	c.(1542 C > T)	T385M	Exon 14	DBD
P5	Male	10	c.(1542 C > T)	T385M	Exon 14	DBD
P6	Female	7	c.(1198 C > G)	L400V	Exon 14	DBD
P7	Female	14	c.(801 T > A)	A267V	Exon 10	CCD
P8	Male	18	c.(1159 A > G)	T387A	Exon 14	DBD
P9	Female	8	c.(1441 G > T)	L351F	Exon 12	DBD

CMCD = chronic mucocutaneous candidiasis disease; aa = amino acid; CCD = coiled-coil domain; DBD = DNA-binding domain.

higher levels of pSTAT1 compared to healthy controls, following both IFNγ stimulation, in $CD14^+$ monocytes ($^{**}p < 0.01$), and IFNα stimulation, in $CD3^+$ lymphocytes ($^{***}p < 0.001$), also confirmed for novel *STAT1* mutations (P1 and P2, P3 and P9, P6). Our results are consistent with data of published studies and confirm for novel heterozygous alleles the association with a dominant phenotype of gain of STAT1 phosphorylation and function.

3.3. L351F and L400V variants lead to a gain of STAT1 function

We analyzed pSTAT1 in Epstein–Barr Virus (EBV)-transformed lymphoblasts of P3 carrying the novel L351F mutation. As illustrated in Fig. 2A, Western blot showed that STAT1 level was normal in patient cells. pSTAT1 had been undetectable in unstimulated cells derived from P1 and P6, and its level had increased in response to both IFNγ- or IFNβ-stimulation (data not shown). In order to prove the gain of function of L351F variant, P3 EBV-transformed lymphoblasts were treated with IFNβ or IFNγ in the presence of the ^{32}P-labeled oligonucleotide STAT-binding probe derived from the IFNγ response region, or type I ISREs. In the presence of both probes, we observed that treatment with IFNs resulted in increased STAT1-binding activity to DNA (Fig. 2B). Moreover, novel mutations L351F (P3 and P9) and L400V (P6) were evaluated by transfection of *STAT1*-deficient U3C fibrosarcoma cells with *STAT1* mutants constructs obtained by site-directed mutagenesis. Responses to cytokine stimulation were investigated by measuring the luciferase activity of the reporter gene under the control of the γ-activated sequence (GAS) promoter. After stimulation with IFNγ or IL27, cells transfected with L351F or L400V alleles responded stronger than those transfected with *wild-type* allele. In addition, stimulation with increasing concentrations of IFNγ (10, 100, 1000 U/ml, respectively) resulted in two to five times stronger responses in cells transfected with novel mutant alleles (Fig. 2D). These results demonstrated how novel heterozygous alleles are GOF for GAS-dependent cellular responses to IFNγ.

3.4. A broad spectrum of infections may manifest beyond chronic candidiasis

3.4.1. Fungal and parasite infections

All patients (mean age 17-year-old, median 14-year-old) presented with recurrent or chronic infection with *Candida albicans* since early childhood (median age at onset < 1-year-old) (See Table 2). Oropharyngeal candidiasis manifested in all cases at onset, 5 patients (55%) developed nail infection by their first decade, 4 patients (45%) had oesophageal candidiasis by their second decade, while skin and genital candidiasis were reported in 2 (P1 and P6) and 1 (P7) patients, respectively. In P4 candidiasis manifested only in a mild form, as oral thrush recurring during antibiotic therapy, well responding to topical antifungal medication (miconazole). Mucocutaneous candidiasis was treated with oral azole antifungal drugs (*i.e.* fluconazole, itraconazole, voriconazole), while treatment of oesophageal candidiasis in P2, P3 and P5 required intravenous antifungal therapy (*i.e.* fluconazole,

Table 2
Clinical and laboratory features of nine patients with STAT1-CMCD.

	Age at onset	Age at diagnosis	CMCD	Treatment resistance	Bacterial Infections	Viral infections	Bronchi-ectasis	Hypo-thyroidism	Other autoimmune manifestations	Lympho-penia	Poor vaccine response
P1	1 yr	9 yrs	+	+	URTI, pneumonias	None	−	+	None	−	−
P2	1 yr	43 yrs	+	+	URTI, pneumonias, skin abscesses	None	+	−	None	+	+
P3	6 mo	28 yrs	+	−	URTI, pneumonias, otitis media, skin abscesses, Leihmaniasis, Cryptococcosis	Recurrent molluscum, skin HPV	+	+	SLE-like skin disease, ANA and ds-DNA antibody positivity	+	+
P4	5 yrs	14 yrs	+	−	URTI, pneumonias	None	+	−	Vitiligo, ANA positivity	+/−	+
P5	2 mo	9 yrs	+	+	URTI, pneumonias, otitis media, sepsis	None	−	−	None	+/−	−
P6	2 yr	7 yrs	+	−	URTI, pneumonias, otitis media	None	+	−	None	−	−
P7	6 mo	14 yrs	+	−	URTI, pneumonias	None	−	−	None	+/−	−
P8	7 yrs	17 yrs	+	−	Parodontitis, skin abscesses, suppurative cyclid infection	Severe chicken pox, recurrent herpetic infections	NK	−	None	−	−
P9	5 mo	8 yrs	+	+/−	Pneumonia, episcleritis	Recurrent zoster and herpetic infections	NK	+	None	−	−

"+" = present; "−" = absent; NK = not known; yr.(s) = year(s); mo = months; CMCD = chronic mucocutaneous candidiasis disease; URTI = upper respiratory tract infections; HPV = human papilloma virus; SLE = systemic lupus erythematosus; ANA = antinuclear antibody; ds = double-strand.

Table 3
Pulmonary findings, current treatment and results of last spirometry of GOF-STAT1 patients.

	Age (yr)	Chronic Lung Disease	Site of bronchiectasis	Age at diagnosis (yr)	Respiratory physiotherapy	Age at starting (yr)	FVC L/%pred	FEV_1 L/%pred	FEV_1/FVC %	FEF 25–75% L/s
P1	11	−	NA	NA	−	NA	2.26/96	2.13/106	94	NK
P2	45	+	L lower lobe, lingula	21	PEP-mask device + short acting bronchodilators, 2 times per day, regularly	30	3.05/88	2.62/84	81	3.42
P3	33	+	L lower lobe, lingula	10	PEP-mask device + short acting bronchodilators, 2 times per day, intermittently	12	3.05/68	2.17/58	71	1.49
P4	15	+	L lower lobe, lingula, R middle lobe	14	PEP-mask device + short acting bronchodilators, 3 times per day, regularly	14	3.54/92	2.66/80	75	2.19
P5	10	−	NA	NA	PEP-mask device + short acting bronchodilators, initially intermittently (*i.e.* during pulmonary infection), then 2 times per day, regularly	4	1.76/92	1.66/98	94	2.72
P6	7	+	L lower lobe, lingula, R middle lobe	6	PEP-mask device + short acting bronchodilators, 2 times per day, regularly	6	NP	NP	NP	NP
P7	14	−	NA	NA	−	NA	2.76/80	2.72/90	99	4.88
P8	18	−	NA	NA	−	NA	NP	NP	NP	NP
P9	8	−	NA	NA	−	NA	NP	NP	NP	NP

"+" = present; "−" = absent; yr. = years; NA = not applicable; NK = not known; L = left; R = right; PEP = positive expiratory pressure; FVC = forced vital capacity; L = liter; pred = predicted; FEV_1 = forced expiratory volume in 1 s; FEF = forced expiratory flow; NP = not performed.

amphotericin B, caspofungin), followed by oral medications (*i.e.* fluconazole, itraconazole, or voriconazole). All patients were started on continuous dosing-regimen: fluconazole was initially the drug of first choice for most of the patients, but changed to itraconazole in P1, P2, P3, and P5 following clinical (persistent or relapsing mucocutaneous candidiasis) and microbiological evidence of resistance. The latter has been monitored with susceptibility testing performed on *Candida* spp. cultured from oropharyngeal swabs, routinely performed in the majority of patients with clinical evidence of infection, and resulting in a minimal inhibitory concentration (MIC) for fluconazole from 8 to 256 mcg/ml in resistant patients. In addition, the majority of patients have been followed with monitoring of serum azole level to concurrently adjust the dosage of medication (target of >500 ng/ml when prophylaxis or >1000 ng/ml when therapy regimen, respectively). We observed, specifically in P2,

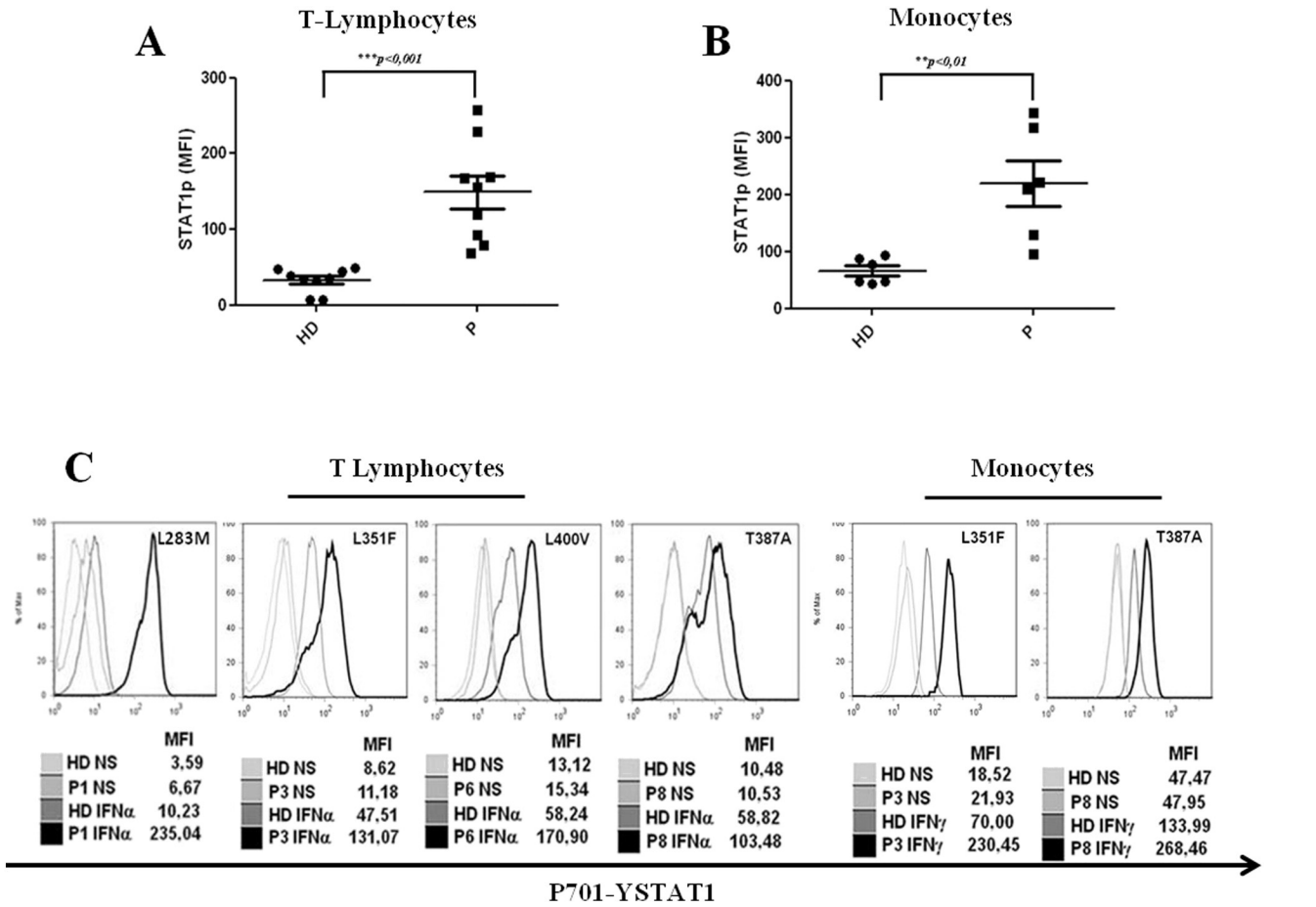

Fig. 1. GOF-STAT1 mutation is associated with increased STAT1 activation. A and B. Summary of mean intensity fluorescence (MFI) of pSTAT1 in T-lymphocytes induced with IFNα (A) and in IFNγ-stimulated monocytes (B). C and D. Intracellular staining of phosphorylated tyrosine 701 STAT1 (P701-YSTAT1) in not stimulated (NS) and IFNα-induced T lymphocytes (C), and not stimulated and IFNγ-induced monocytes (D). Each experiment was independently repeated at least three times for each patient: representative cases are shown. MFI = mean intensity fluorescence; HD = healthy donor.

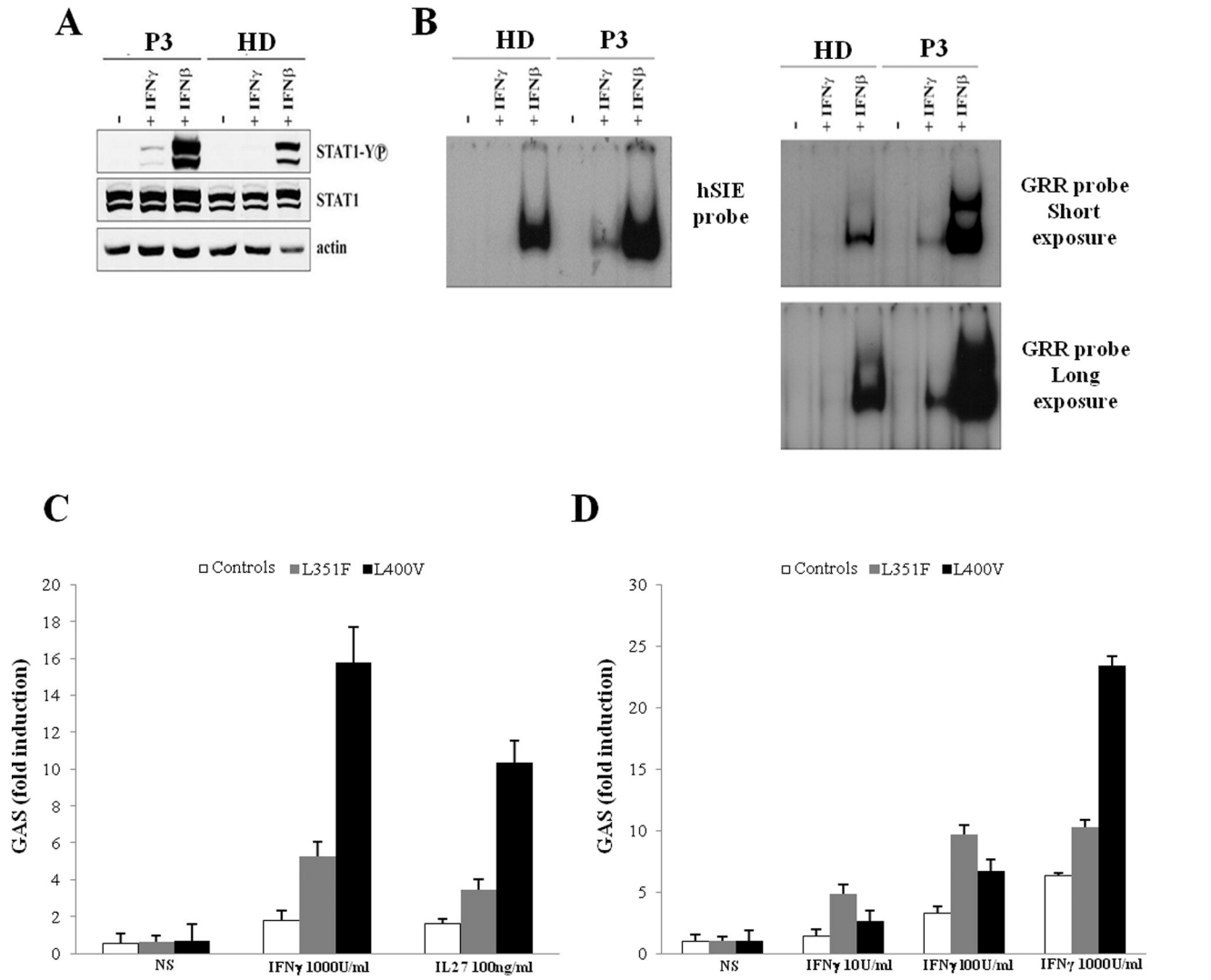

Fig. 2. Novel mutants L351F and L400V *STAT1* alleles are dominant for GAS-dependent cellular responses. A and B. Response of P3 EBV-B cells (L351F) by Western blot (A) and EMSA (B). C and D. Induction of GAS-dependent reporter gene transcription activity in U3C cells transfected with WT or mutant alleles of *STAT1* (L351F and L400V): cells were stimulated with IFNγ or IL27 (C) and increasing doses of IFNγ (D). Results derived from one representative experiment of three independent experiments. HD = healthy donor; NS = not stimulated.

suffering the most severe form of chronic mucocutaneous candidiasis (*i.e.* oropharynx, nails, esophagus), that intermittent regimen treatment and use of suboptimal dosage predisposed to persistent infection and development of resistance to azoles (initially limited to fluconazole, but extended to itraconazole and voriconazole, with current sensitivity for posaconazole). Interestingly, in addition to *Candida* infection, P3 also developed an intracellular pathogen infection with *Cryptococcus neoformans* (Fig. 3), presenting as disseminated granulomatous necrotizing lymphadenitis at the age of 14 years, well responding to intravenous fluconazole. Moreover, at the age of 28 years, the same patient suffered from disseminated visceral leishmaniasis, was initially treated with amphotericin B, but the latter caused drug-related nephropathy, and was replaced with miltefosfine.

3.4.2. Bacterial infections

The majority of patients (no relevant episodes in P8 history) had a history of recurrent upper respiratory tract infections and experienced pneumonias (See Table 2), variably associated with bouts of obstructive bronchitis, since their childhood, often requiring hospitalization for intravenous antibiotic therapy. *Haemophilus influenzae*, *Streptococcus pneumoniae*, or *Staphyloccocus aureus* were the pathogens usually detected in the sputum, when culture was available. Moreover, some patients had recurrent pulmonary exacerbations with opportunistic pathogens. P2, P4 and P6 had been suffering from intermittent infection with *Pseudomonas aeruginosa*; in P4 bronchopneumonia with *Serratia marcescens* recurred twice when he was 14-year-old. The recurrence of pulmonary infections has currently led to permanent lung damage in 4 patients of our cohort (P2, P3, P4, P6), as diagnosed by chest computed tomography (CT)-scan (Pulmonary findings summarized in Table 3; see Fig. 2 in [16]). All patients had been commenced on pneumonologist follow-up, together with regular respiratory physiotherapy, in order to enhance clearance of bronchial secretion and control pulmonary exacerbations [21–23]. The analysis of spirometry results [24] in these patients developing chronic lung disease shows a tendency to develop obstructive pulmonary disease, with a forced expiratory volume in 1 s (FEV_1) generally below normal range, associated to a reduction in the forced expiratory flow during the interval 25–75% of the forced vital capacity (FEF 25-75%). These changes of FEF 25-75% constitute a sensitive parameter in the detection of obstructive small airway disease [25]. Specifically, P4 has experienced a rapid decline of his lung function (Forced Vital Capacity -FVC- from 3.17 to 2.17 L, FEV_1 from 2.35 to 1.68 L, and FEF 25-75% from 1.78 to 1.49 L/s) in a eight-months time characterized by pulmonary exacerbations with *Serratia*: in this patient, in few months, we have currently observed a return of his pulmonary tests to normal values (FVC 3.54 L, FEV_1 2.66 L, FEF 25-75% 2.19 L/s) and no pulmonary exacerbations, likely related to the increase of daily

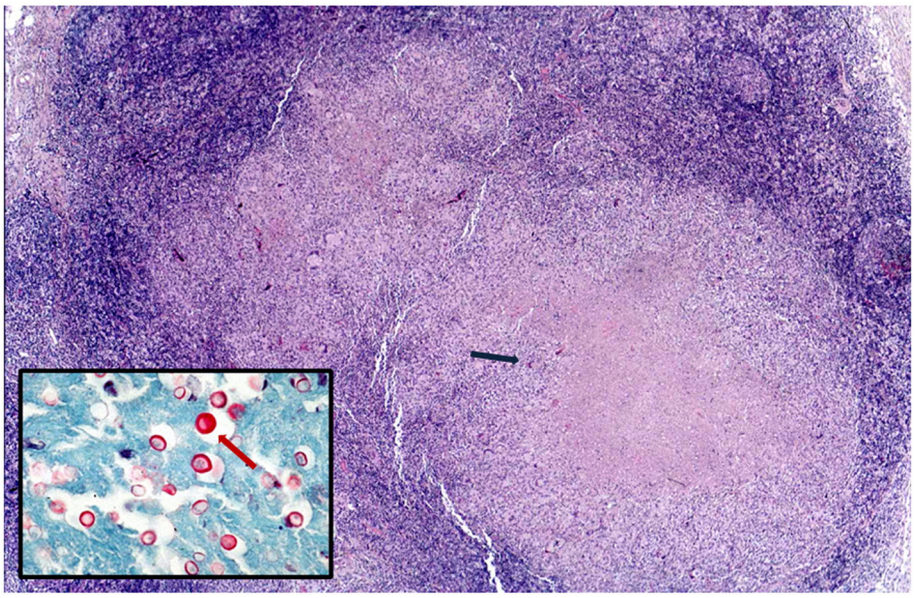

Fig 3. Necrotizing granulomatous lymphadenitis (blue arrow), containing numerous mucicarmine-positive cryptococci (red arrow) (Hematoxylin-Eosin; inset: mucicarmine stain). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

respiratory physiotherapy and the start of antibiotic prophylaxis with azithromycin, following the rationale of a reduction of pulmonary exacerbations in patients with non-cystic fibrosis bronchiectasis by using low-dose macrolide [26].

Among other bacterial infections, our cohort features abscesses and sepsis. Three patients (P2, P3, and P8) suffered from skin abscesses: in P3 a coagulase negative *Staphylococcus* was detected and infection resolved after intravenous antibiotic and surgical excision, while P8 had recurrent *S. aureus*-positive abscesses requiring systemic antibiotic treatment, and P2 was alternately treated with oral or intravenous antibiotics for recurrent boils. P5 had a *Streptococcus viridians* sepsis, concomitantly to a *Pneumocystis jirovecii* pneumonia, at the age of four months (lymphocyte subsets had been performed few months after the acute episode with normal results), while P2 sepsis was caused by Difteroides spp. when 28-year-old.

3.4.3. Viral infections

We observed abnormal response to viral infections in some patients (See Table 2). P8 had varicella with severe involvement of mucosa at the age of 8 years. Both P8 and P9 presented with recurrent mucocutaneous infections with herpes simplex viruses and severe episodes of varicella-zoster infection. P3 had molluscum and human papilloma virus cutaneous lesions.

3.5. Autoimmune manifestations may be part of a heterogeneous phenotype

In our cohort, hypothyroidism manifested in three patients (P1, P3, and P9; see Table 2): all cases had negative auto-antibodies (antithyroglobulin, antithyroid peroxidase, anti thyroid-stimulating hormone -TSH- receptor antibody), but typically presented a dysomogeneous ultrasound appearance of the gland. All patients started the treatment of hypothyroidism with levothyroxine by their first decade of life. On autoimmune screening, antinuclear antibodies tested positive in P3 (title increasing from 1:160 to 1:320) and P4 (stable title 1:320), in P3 also associated to positivity for anti-double strand DNA antibodies (138–122%, normal value <35%): P3 has developed Systemic Lupus Erythematosus (SLE)-like skin face lesions, while P4 suffers from a mild form of vitiligo. Despite cerebral vasculitis and cerebral and aortic aneurysms have been reported [19], none of our patients ever had signs or symptoms suggesting investigation for cerebral or heart complications.

3.6. The immunological phenotype: risk of progressing lymphopenia

Analysis of lymphocyte subsets in each patient of our cohort (Fig. 4; see Table 1 in [16]) revealed persistent lymphopenia in two adult patients. In P2, total lymphocytes range from a maximum of 1250 cells/mmc to 810 cells/mmc (mean below 1000 cells/mmc), with low counts of both T and B lymphocytes (normal $CD4^+/CD8^+$ ratio and NK cell counts in the normal range). P3 developed lymphopenia in his twenties, with total lymphocytes ranging from 700 to 1130 cells/mmc, low numbers of $CD3^+$ (375–730 cells/mmc), $CD4^+$ (233–421 cells/mmc), and $CD8^+$ (128–272 cells/mmc) cells, but normal B cell count (261–335 cells/mmc). Interestingly, P3 also showed low NK cells count, that was persistently below 50 cells/mmc (11–44 cells/mmc). In P4 we observed transitory reduction of $CD4^+$ T cells (382 cells/mmc), out of a specific infectious episode, in P5 low $CD4^+$ T cells (1153 cells/mmc) during his first year of life, but out of his episode of sepsis, and in P7 a single determination of low $CD19^+$ cells (152 cells/mmc). Moreover, lymphocyte subsets analysis in six patients showed a low percentage of memory B cells, with reduction of both un-switched (median 3% of B cells) and switched subsets (median 1% of B cells) (see Table 1 in [16]).

Three patients (P2, P3, P4) also had defective antibody response to tetanus toxoid (<0.05 UI/ml), with unprotective title confirmed after booster vaccine. Overall, serum immunoglobulin levels, TRECs, and lymphocyte proliferation assays to mitogens did not show abnormalities (data not shown). We observed low immunoglobulin (Ig) M level only in P1 in different determinations (IgM 31 mg/dl -range 56–261- at the age of 6 year-old; IgM 46 mg/dl -range 61–276- at the age of 10), with normal IgG (1040 mg/dl -range 707–1919-) and IgA (143 mg/dl -range 60–270-).

4. Discussion

4.1. Novel mutations confirm the increased STAT1 phosphorylation

We describe nine patients of Caucasian origin and Italian nationality with heterozygous GOF mutations in *STAT1*. Three novel mutations were identified: L283M in the CCD, while L351F and L400V in the

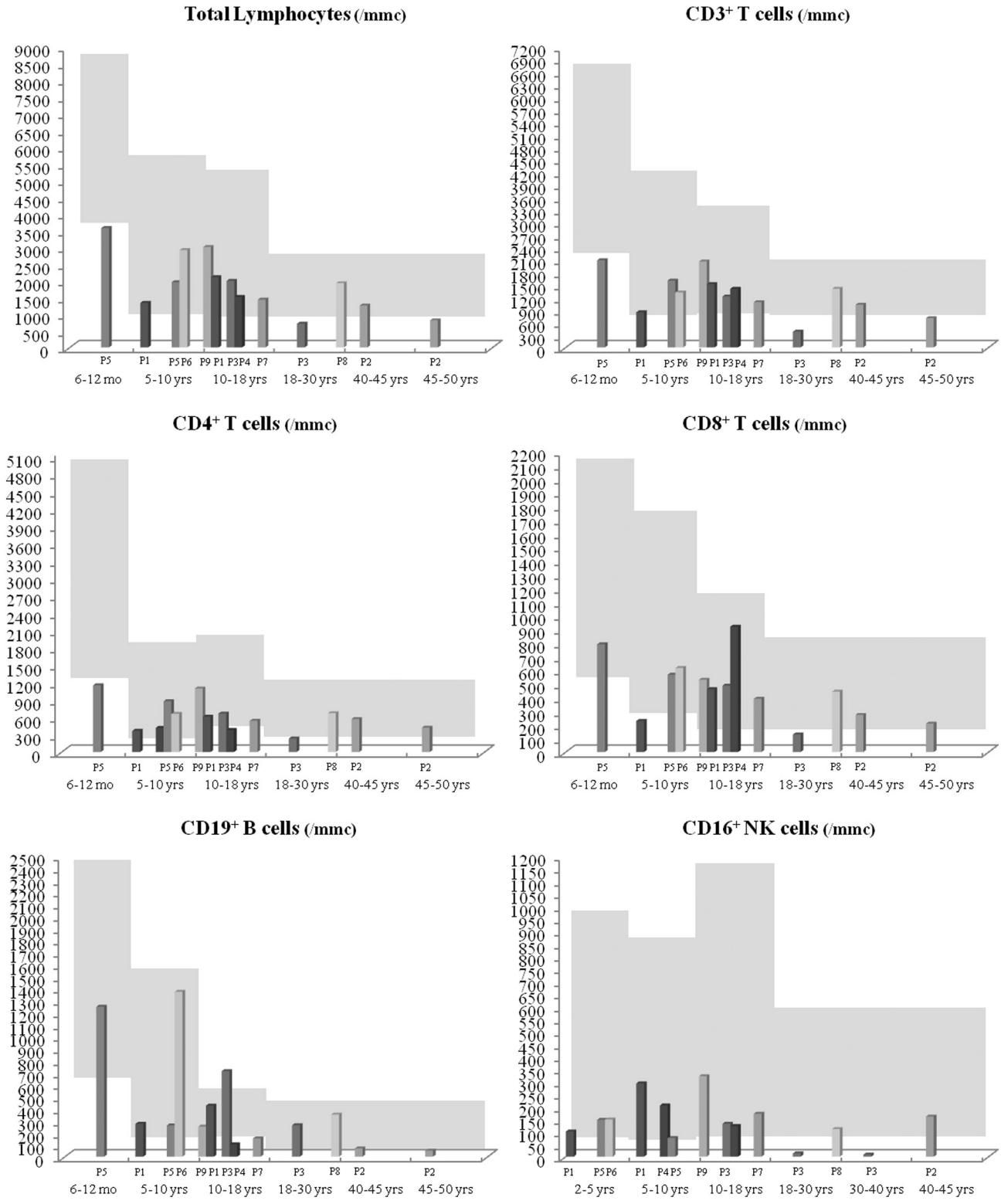

Fig. 4. Lymphocyte subsets for each CMCD patient are shown as a single value per age range. Normal ranges of lymphocyte counts, we have illustrated in the chart background, were based on age-matched healthy control subjects. The complete series of lymphocyte counts is shown in the Table 1 in [16].

DBD. The underlying pathogenesis involves a gain of STAT1 phosphorylation resulting in a gain-of-function of GAF in response to various cytokines. Consistently with previous reports, levels of pSTAT1 are high in $CD3^+$ and $CD14^+$ cells, gamma-activated sequence binding activity increases following stimulation with IFNγ, and cellular response is enhanced to cytokines such as IFN-α/β, IFN-γ, and IL-27, which potently inhibit development of IL-17–producing T cells *via* STAT1 [1–3,27].

4.2. CMCD represents the hallmark of GOF-STAT1 phenotype with high risk of resistance to treatment

In the clinical phenotype of the majority of patients, CMCD represents the main manifestation and feature of suspicion at onset, but severity largely varies from chronic recurrent and debilitating infection to milder form, detectable after detailed questioning of medical history,

or occurring only during antibiotic therapy. We show high risk of developing resistance to oral antifungal treatment, associated to high MIC to azoles (*i.e.* fluconazole), and resulting in chronic infection with *Candida*. Development of antifungal resistance in our patients was not associated to any specific STAT1 mutations. Conversely, STAT1 patients displaying mild forms of candidiasis may suggest that genetic or environmental factors could contribute to their susceptibility to fungal infections. In particular, intermittent regimens of antifungal treatment or use of azoles at suboptimal doses might favor the selection of azole-resistant strains of *Candida*. Development of azole-resistance may require use of alternative regimens including caspofungin, or liposomal amphotericin B, that are administered by an intravenous route. In addition, case report studies have suggested that immunomodulatory treatment with cytokines such as granulocyte-colony stimulating factor (G-CSF) or granulocyte monocyte-colony stimulating factor (GM-CSF) may restore the generation of Th17 cells and parallel enhance recovery from fungal infections [28]. However, these preliminary findings should be confirmed in larger cohort of patients. Until then, our study suggests that antifungal prophylaxis with fluconazole or itraconazole should be commenced in all patients at the time of diagnosis, regularly continued and strictly monitored by pharmacokinetics measurements and susceptibility testing, in order to optimize control of diffuse candidiasis, prevent developing of inadequate dose-related resistance, and guarantee efficient treatment.

4.3. A broader spectrum of infections, the occurrence of chronic lung disease, lymphopenia, or autoimmunity may complicate the prognosis

GOF-STAT1 patients may typically experience also recurrent bacterial infections since early childhood, and present viral diseases mainly with herpes family viruses [12,13,15,29], causing high morbidity and increasing the mortality risk. Specifically, Sherfe *et al.* reported of three patients who died of overwhelming viral infections (Cytomegalovirus, Epstein–Barr virus, and Jamestown Canyon virus, respectively), interestingly presenting with a combined immunodeficiency (CID)-like immunological phenotype (progressive loss of T cells and natural killer cells, plus humoral defect). In our cohort, we observed that lymphopenia, manifesting with reduction in $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes subsets, may not be constantly present, but might progress by adulthood. Consistent with that, careful monitoring of main lymphocyte subsets could help in clinical management by early detection of patients developing severe lymphopenia that might require to be evaluated for hematopoietic stem cell transplantation (HSCT). Interestingly, we report a reduction in both un-switched and switched memory B cells in the majority of our patients, supporting a previous observation in the case report by Frans *et al.* [30]. Currently, any genotype–phenotype correlation seems consistent, even though we observed same characteristic features in patients with same mutations. Particularly, in our cohort an increased susceptibility to viral infections manifested in patients carrying L351F and T387A variants. Meanwhile, we observed how T385M variant differently manifested in two patients with similar age and sex: P4 suffered from severe and recurrent lower respiratory tract infections and developed bronchiectasis and chronic lung disease in his adolescent age; P5 presented with early severe reflux disease, required Nissen fundoplicatio, and had jatal hernia, concurrently with oesophageal candidiasis. Similarly, in previous reports [30], this mutation variably presented with mainly gastrointestinal or respiratory phenotype, but also with progressing combined immunodeficiency predisposing to fatal infections [15,19,31]. For other novel mutations herein identified, major phenotypic manifestations include early development of bronchiectasis in patient carrying the L400V variant, and lymphopenia and chronic lung disease progressing by adulthood in the familiar variant L283M. These phenotypes may add new characteristic manifestations, but are insufficient to hypothesize any certain correlation with the genotype; indeed, the paucity of reported cases likely suggests any observed association still as serendipity. More important, we show how lung disease represents a major complication as it may slowly evolve by adulthood to permanent lung damage predisposing to recurrent pulmonary exacerbations and higher morbidity. Early recognition of lung damage at initial stage associated to prompt aggressive treatment of pulmonary infection and daily respiratory physiotherapy might prevent progression of the disease. The role of antibiotic prophylaxis, including low-dose macrolide, could be controversial because of increased risk of progressing candidiasis. Finally, autoimmune manifestations represent common features of the disorder and extensive autoimmune assessment should be regularly performed: consistently with what previously reported, thyroidopathy represents the most common disorder [1,19], variably onsets by the first or second decade of life, associates to no thyroid antibody or significant alterations of B subsets, but gland abnormalities, supporting the hypothesis of an increased response to type I IFNs and the role of IFNs signature in the disease [3,32]. Recently, Oftedale *et al.* redefined a "non-classical" form of autoimmune polyendocrine syndrome type 1 (APS-1) where a milder, less penetrant autoimmune phenotype associates to dominant heterozygous mutations in AIRE [33]. Even though AIRE p.R257 mutation is known to behave in a recessive manner, further characterization could be pursued to rule out the role of AIRE mutation in our patient P3 who features CMCD together with a mild autoimmune phenotype.

5. Conclusion

GOF-STAT1 disorder represents an emerging primary immunodeficiency and analysis of larger cohort of patients is warranted to better characterize clinical and genetic heterogeneity, long-term complications, and prognosis of the disease.

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2.4 Impaired NK cells functions in patients with STAT1 gain-of-function mutations

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Introduction

The identification of STAT1 GOF mutations have contributed to reveal the role of Th17 cells in the immune response against Candida infections (110). Th17 cells are important for the defense against microbial infections at the mucosal barrier and secrete IL-17F, IL-17A, IL-22, and IL-21. In several PIDs the evidence of CMC has been associated with impaired Th17 development (59). Increased and persistent phosphorylation of STAT-1 in CMCD results in impaired response to the cytokines that promote Th17 differentiation (73, 81). STAT1 act downstream type I IFNs and other cytokines regulating the transcription of genes involved in the response to viral infections (111). Previous studies revealed the importance of STAT1 signaling in the activation of Natural killer (NK) cells (112, 113) which play an important role in the immune response against intracellular pathogens and virally infected cells (114). STAT1 expression and phosphorylation are induced during early viral infections. In response to stimulation with type I IFNs, NK cells acquire effector functions and secrete immunomodulatory cytokines (i.e. IL-15), that regulate NK cell expansion (113).

Apart from fungal infections at the mucosal barrier CMCD patients may also present viral infections, including recurrent mucocutaneous infections due to reactivation of varicella-zoster and herpes simplex viruses, debilitating orf infection, or severe invasive infection due to chicken pox, cytomegalovirus (CMV) or Epstein-Barr virus (EBV) (88, 90, 93, 94). These observations suggest that also NK cells might play a role in the pathogenesis of CMCD.

In this study, submitted to *Journal of Allergy and Clinical Immhnology* as *Full Paper*, we investigated STAT1 and STAT5 signaling in response to specific cytokines, cytotoxic activity, cytokine production and proliferation in NK cells from eight CMCD patients. We observed that STAT1-GOF mutations result in increased STAT1 phosphorylation, and reduced STAT5 activation, in response to IL-2 and IL-15. These alterations lead to impaired proliferation in response to IL-2 or IL-15, and to reduced IFN-γ secretion in response to IL-15.

Results

Eight patients with gain-of-function STAT1 mutations were included into the study (Table XIII).

Table XIII. Genetics and main phenotypical features of eight patients with gain-of-function STAT1 mutations

	P1	P2	P3	P4	P5	P6	P7	P8
Mutation (c.DNA)	c.(847T>A)	c.(847T>A)	c.(1441G>T)	c.(1542C>T)	c.(1542C>T)	c.(1198C>G)	c.(801T>A)	c.G372C
Mutation (aa)	L283M	L283M	L351F	T385M	T385M	L400V	A267V	T387A
Affected Domain	CCD	CCD	DBD	DBD	DBD	DBD	CCD	DBD
Sex	Male	Female	Male	Male	Male	Female	Female	Male
Age	11	45	33	15	10	7	14	18
Clinical onset	1 year	1 year	6 months	5 years	2 months	2 years	6 months	7 years
Manifestations of CMC	Oral cavity, skin, nails	Oral cavity, nails	Oral cavity, esophagus	Oral cavity	Oral cavity, oesophagus	Oral cavity, skin, nails	Oral cavity, genital mucosa	Oral cavity, nails, esophagus
Infections	URTI, pneumonia	URTI, pneumonia, skin abscess	URTI, pneumonia, otitis media, skin abscess, Cryptococcal adenitis, leishmaniasis, skin HPV, recurrent mollusca	URTI, recurrent pneumonia	URTI, *Pneumocystis* pneumonia, otitis media, sepsis	URTI, pneumonia, otitis media	URTI, pneumonia	Skin abscesses, parodontis, suppurative eyelid infection, mucocutaneous HSV infection, severe chicken pox
Inflammation	None	Reflux esophagitis, recurrent fever, chronic lung disease, bronchiectasis	Recurrent fever, chronic lung disease, bronchiectasis	Bronchiectasis, anemia	GERD	Bronchiectasis, anemia	None	None
Endocrinopathy	Hypothyroidism	None	Hypo-thyroidism	None	None	None	None	None
Autoimmunity	None	None	Lupus-like skin lesion, ANA and ds-DNA antibody positivity	ANA positivity, vitiligo	None	None	None	None
Lymphopenia	No	Yes	Yes	Yes	Yes	No	No	+/-
Vaccine response	Normal	↓	↓	↓	Normal	Normal	Normal	Normal

NK functional activity and phenotype in CMCD patients

NK cytotoxicity was evaluated in three CMCD patients and three healthy subjects by using a chromium release assay. A significant reduction of cytolytic activity of cells from CMCD patients as compared to normal controls was observed (Figure 4, A and B). However, the evidence of the broad variability in the number of NK cells observed in CMCD patients might account for the impaired cytotoxicity of cells. Therefore, the cell surface upregulation of the degranulation marker CD107a on CD56+CD3- was evaluated by flow cytometry after exposure to the NK cell susceptible human erythroleukemia K562 cell line, before and after overnight culture in the presence of IL-2. A reduction of the degranulation activity of resting NK cells as compared to healthy controls was observed in CMCD ($p>0.05$). Similarly, a significant reduction of degranulation activity as compared to healthy subjects was observed

in IL-2 activated NK cells ($p<0.05$) (Figure 4, C and D). Intracytoplasmic perforin content was normal in NK cells from patients with STAT1-GOF mutations. The analysis of NK-cell subsets, on the basis of CD56 and CD16 expression and the pattern of activating and inhibitory receptors on NK cells revealed a reduced expression of CD57 on CD56dullNK cells in 3 out of 9 CMCD patients, while KIR and NKG2A molecules were expressed at normal levels. Both CD56bright and Cd56dim subsets of NK cells showed normal expression of NKG2D, the activating NK receptors NKp46 andNKp30, and several other co-receptors(115). Likewise, the expression profile of the chemokine receptors CXCR1 and CCR7 of the two CD56 subsets were normal.

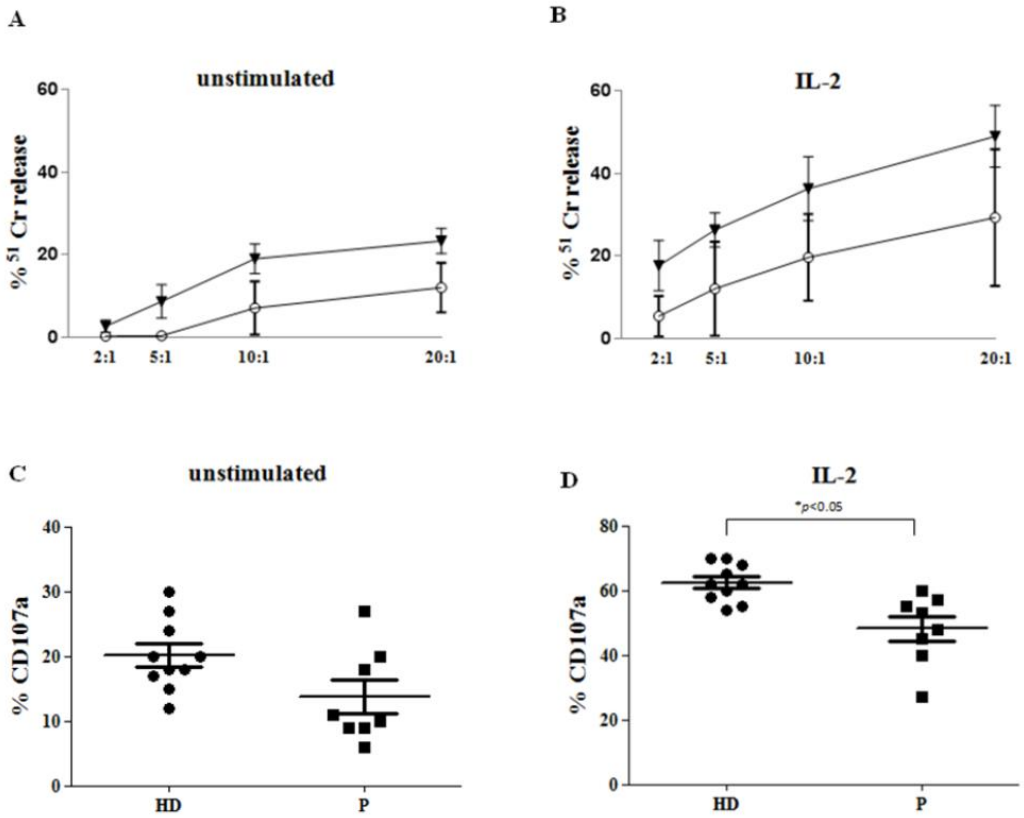

Figure 4: Impaired NK cell cytotoxicity in CMCD patients. (A and B) Freshly isolated PBMCs derived from three control subjects (triangle) and from three patients (circle) were tested against the K562 target cells line overnight incubation, either without IL-2 (A) or with IL-2 (B) at different E/T ratios. Data from the control group show the mean ± SD of three replicates (C and D). Degranulation assay (as measured by CD107a expression) of freshly CD56+ CD3- gated NK cells derived from eight CMCD patients (square) and ten healthy donors (circle) after stimulation with the human K562 cell line. PBMCs were incubated overnight without (C, $p>0.05$) or with IL-2 (D, $p<0.05$).

STAT1 and STAT5 phosphorylation in NK cells of CMCD patients

STAT1 and STAT5 phosphorylation in response to IFN-α (40000U/ml for 30 minutes), IL-2 (100ng/ml for 12 minutes), IL-15 (50ng/ml for 12 minutes), or IL-21 (50ng/ml for 15 minutes) was evaluated by flow cytometry in NK cells from healthy controls and CMCD patients. Increased STAT1 phosphorylation in response to IFN-α as compared to control values was observed in CD56+CD3- NK cells of CMCD patients, suggesting that STAT1-GOF mutations result in abnormal STAT signaling also in NK cells (Figure 5 A). The study of STAT1 phosphorylation in IL-2 activated NK cells revealed increased STAT1 phosphorylation in response to both IL-2 and IL-15 in NK cells from CMCD as compared to healthy controls (Figure 3, B and C, $p<0.05$). STAT1 protein expression was analyzed in order to investigate the mechanism leading to the increased STAT1 phosphorylation. Up to three fold increase of protein levels in resting CD56+CD3- NK cells from CMCD patients as

compared to those from healthy control individuals was observed by flow cytometry (Figure 5 D).

Western blot analysis of STAT1 in IL-2 activated NK cell from the patient P7 revealed an increase of STAT1 protein levels of about three fold in the cells from the CMCD patient (Figure 5 E, left panel). This is accord with the analysis of STAT1 expression by flow cytometry in the same patient (Figure 5 E, right panel). IFN-α induced higher level of pSTAT1 in IL-2 activated NK cells from patient P7 as compared to cells from the healthy control. A strong STAT1 phosphorylation was observed, in the same patient, also in response to IL-2 and IL-15 stimulation (Figure 5 F). Analysis of STAT1 phosphorylation in response to IL-21 did not reveal STAT1 activation neither in control nor in cells from the CMCD patient.

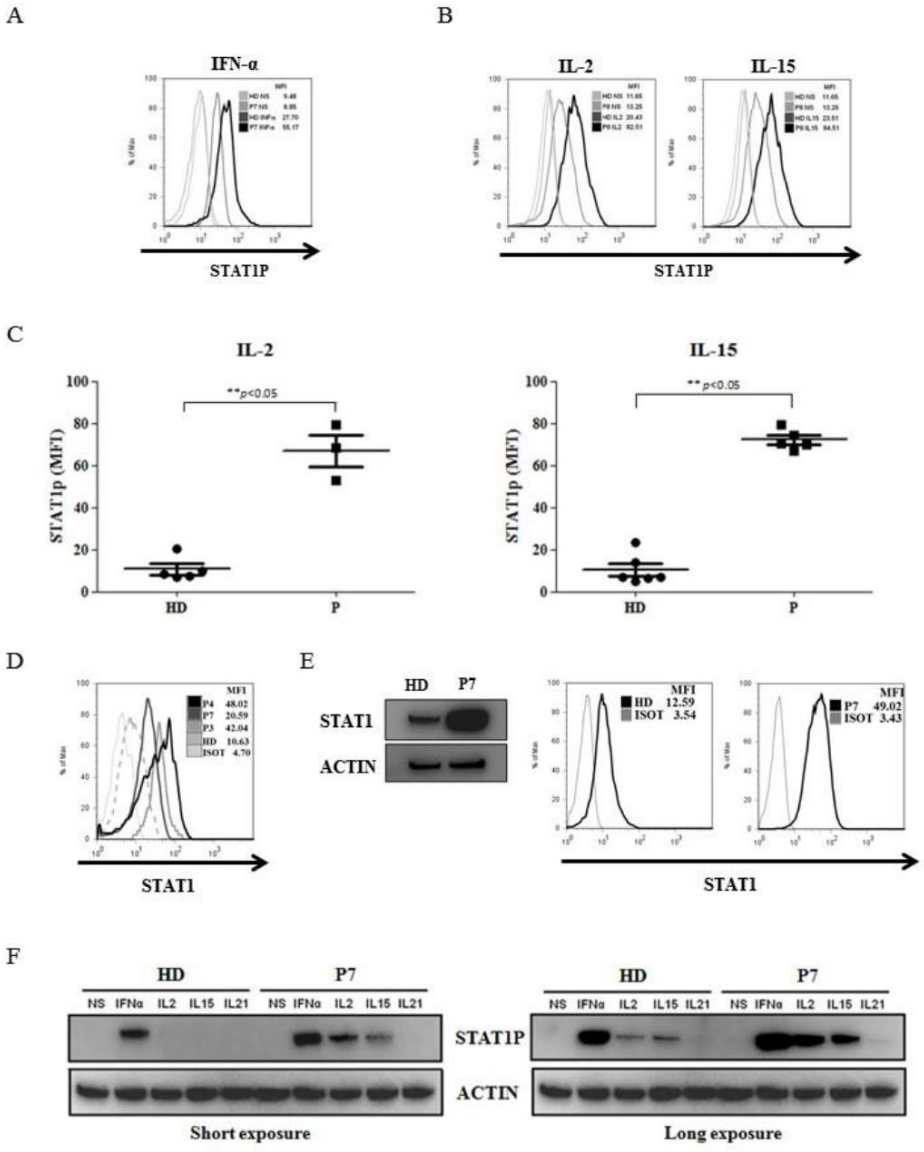

Figure 5: GOF-STAT1 mutations in CMCD patients are associated with increased STAT1 activation. (A) NK cells derived from three CMCD patients (P3, P7, P8) and three healthy controls were stimulated with IFN-α (40000U/ml) for 30 minutes, or were left unstimulated. Cells were stained with anti-CD3, anti-CD56, anti-pY701 STAT1 and analyzed by flow

cytometry after gating CD56+CD3- cells. The extent of pSTAT1 intensity, expressed as Mean Fluorescence Intensity (MFI), of a representative patient is shown. (B) NK cells from five patients (P3, P5, P6, P7, P8) and six healthy controls were stimulated with 100ng/ml IL-2, or 50ng/ml IL-15 for 12 minutes, or were left unstimulated. Cells were stained with anti-CD3, anti-CD56, anti-pY701 STAT1 antibodies and analyzed by flow cytometry after gating on CD56+CD3-. A representative patient is shown. The extent of pSTAT1 expression is calculated as MFI. (C) STAT1 activation in NK cells derived from CMCD patients (square) and healthy donors (circle) after stimulation with IL-2 or IL-15. The extent of STAT1 phosphorylation was calculated as MFI after subtracting the MFI level of unstimulated cells stained with an isotype-matched mAb. Mann-Whitney U-test statistical analysis shows a significant difference in the extent of STAT1p expression between CMCD patients and control subjects ($p<0.05$). (D) NK cells derived from three CMCD patients (P3, P4, P7) and healthy control were stained with anti-CD3, anti-CD56, and anti-STAT1 and analyzed by flow cytometry after gating CD56+CD3- cells. The extent of STAT1 intensity is expressed as MFI. (E) STAT1 expression in NK cells from a healthy control subject (HD) and patient P7 was evaluated by Western blot (panel left) using an anti-STAT1 mAb. Actin was used as a loading control reference. STAT1 and actin expression in NK cells were calculated by densitometric analysis; STAT1/actin expression ratio was calculated in cells from the CMCD patient and control subject. STAT1 protein expression was also evaluated also by flow cytometry (right panel) using (PE)-conjugated mouse anti-STAT1. The extent of STAT1 expression is calculated as MFI. (F) Analysis of STAT1 phosphorylation by Western blot. NK cells from a healthy control subject (HD) and from patient P7 were stimulated with IFN-

α (10000U/ml) for 30 minutes, IL-2 (100ng/ml) for 12 minutes, IL-15 (50ng/ml) for 12 minutes, IL-21 (50ng/ml) for 15 minutes, or medium alone. STAT1 phosphorylation was evaluated with an anti-Tyr-701-phosphorylated STAT1 mAb. Analysis of actin expression was used to measure protein loading. Both long or short film exposure are shown in figure.

The analysis of STAT5 phosphorylation of IL-2-activated NK cells by flow cytometry revealed reduced pSTAT5 levels in all CMCD patients tested in response to both IL-2 and IL-15 as compared to healthy control cells (Figure 6, A and B , $p < 0.05$). On the contrary, the expression of STAT5 was comparable in resting CD56+CD3- NK cells from both the patients and healthy control (Figure 6 C). In order to assess the expression levels of STAT5, a western blot analysis was carried out (Figure 6 D). WB analysis on NK cell cultures derived from patient P7 and from a healthy donor after withdrawal of IL-2 showed similar levels of STAT5 expression. Conversely, WB analysis of STAT5 phosphorylation in NK cells from patient P7 showed a decrease, by approximately fifty percent, of pSTAT5 levels (Figure 6 E).

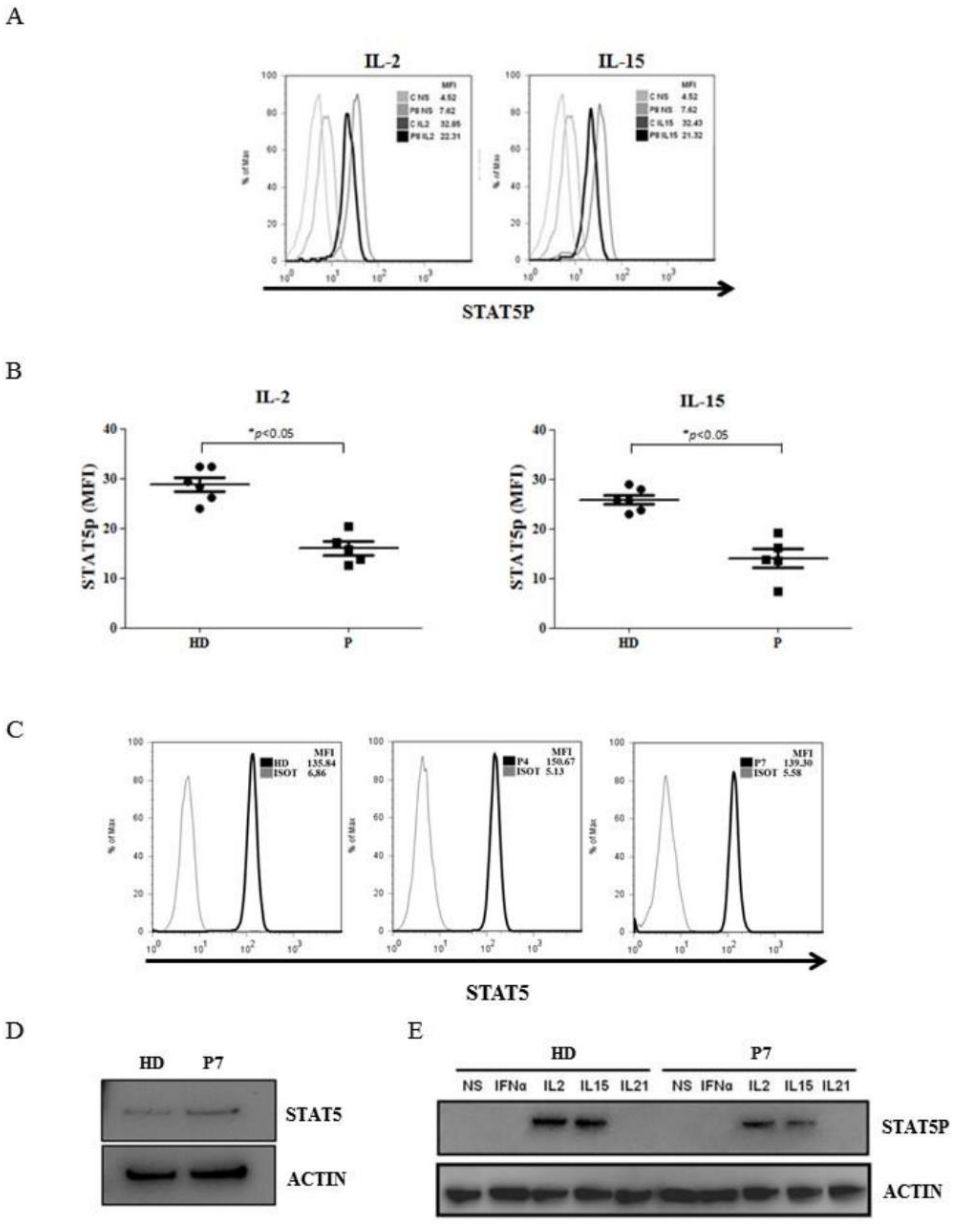

Figure 6. GOF STAT1 mutation in CMCD patients are associated with reduced STAT5 activation. (A) NK cells from five CMCD patients (P3, P5, P6, P7, P8) and six healthy controls were stimulated with IL-2 (100ng/ml) or IL-15 (50ng/ml) for 12 minutes, or were

left unstimulated. Cells were stained with anti-CD3, anti-CD56, anti-pY694 STAT5 antibodies and analyzed by flow cytometry after gating CD56+CD3-cells. A representative case is shown. The extent of pSTAT5 expression is calculated as MFI. (B) STAT5 activation in NK cells derived from CMCD patients (square) and healthy donors (circle) after stimulation with IL-2 or IL-15. The extent of STAT5 phosphorylation was calculated as MFI after subtracting the MFI level of unstimulated cells stained with an isotype-matched mAb. Mann-Whitney U-test statistical analysis shows a significant difference in the extent of pSTAT5 expression between CMCD patients and control subjects ($p<0.05$). (C) NK cells derived from CMCD patients P4 and P7 and healthy control were stained with anti-CD3, anti-CD56, and anti-STAT5 and analyzed by flow cytometry after gating CD56+CD3- cells. Panel includes STAT5 intensity as Mean Fluorescence Intensity (MFI). (D) Western blotting analysis of STAT5 expression was carried out on unstimulated NK cells derived from healthy control subject (HD) and from patient P7. Actin expression was evaluated as a loading control reference. STAT5 and actin expression in NK cells were calculated by image densitometry analysis; STAT5/actin expression ratio was calculated in cells from the CMCD patient and from the control subject. (E) Analysis of STAT5 phosphorylation by Western blot. NK cells derived from healthy control subject (HD) and from patient P7 were stimulated with IFN-α (10000U/ml) for 30 minutes, IL-2 (100ng/ml) for 12 minutes, IL-15 (50ng/ml) for 12 minutes, or IL-21 (50ng/ml) for 15 minutes, or medium alone. STAT5 phosphorylation was investigated by using an anti-Tyr-694-phosphorylated STAT5 mAb. Analysis of tubulin expression was determined to measure protein loading.

Abnormal STAT1 and STAT5 DNA binding activity in NK cells of CMCD patient

A chromatin immunoprecipitation (ChIP) assay in NK cells from patient 7 and in cells from a control subject was performed in order to evaluate the direct binding of STAT1 and STAT5 to specific genomic regions containing the canonical ISRE binding site or the STAT5 binding motif such as the IFIT1 and the IL2RA promoters (116, 117) (Figure 7). In unstimulated NK cells from the CMCD patient we identified STAT1 binding to the IFIT1 promoter, probably due to the presence of a constitutively activated form of STAT1 in these cells (Figure 7 A). Moreover, IL-15 stimulation of NK cells from the patient induced a stronger recruitment of STAT1 to the IFIT1 promoter as compared to NK cells from healthy donor (Figure 7 A). Conversely, STAT5 binding to IL2RA, a gene strictly dependent on STAT5, was increased by IL-15 stimulation only in NK cells from the healthy donor, but not from the patient (Figure 7 B), in accordance with the low level of phosphorylation of STAT5 observed in IL-15 stimulated NK cells from patients (Figure 6).

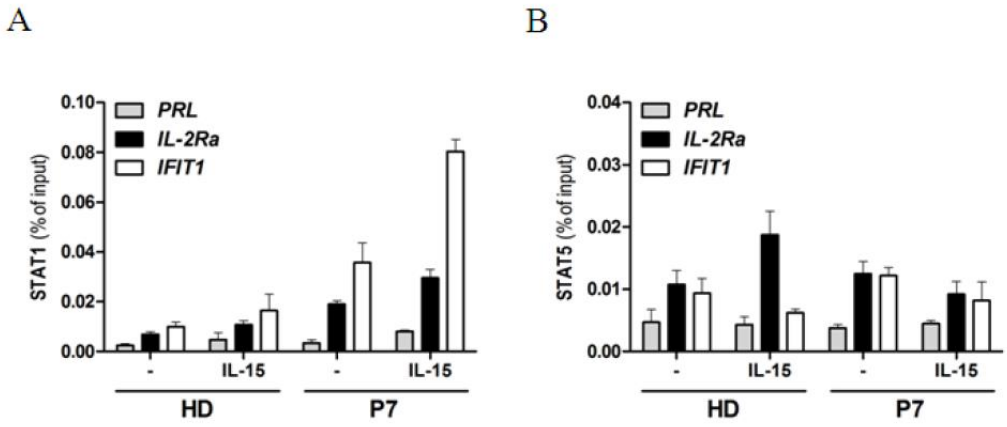

Figure 7. Abnormal STAT1 and STAT5 DNA binding activity in IL-15 stimulated NK cells. (A and B) NK cells derived from healthy control subject (HD) and from patient P7 were stimulated with IL-15 (50ng/ml) for 45 minutes, or left unstimulated. ChIP assay was then performed using STAT1 (A) and STAT5 (B) Abs; co-immunoprecipitated DNA samples were amplified by IL2RA, IFIT1 and PRL promoters specific primers. Data are expressed as percentage of the total input. A representative experiment of two performed is shown.

Gene expression in NK cells of CMCD patient

Real Time PCR was performed in NK cells activated with IL-2 from patient P7 and from a healthy control to address the effects of GOF STAT1 mutation on gene expression. STAT1 mRNA leves were already elevated in unstimulated cells of the patient as compared to the healthy control, thus explaining the finding of increased STAT1 protein levels in NK cells from the same patient. After stimulation with IL-2 and IL-15 a further increase of mRNA expression was observed in the patient as compared to the control (Figure 8 A). The expression of IL2RA mRNA was evaluated since the cells of patients show a decrease in phosphorylation of STAT5 after stimulation with IL-2 and IL-15. A greater increase in the expression of IL2RA mRNA was reported in the patient's cells after treatment with IL-2 or IL-15cells as compared to the control (Figure 8 B). SOCS3 mRNA upregulation was observed in the patient, but not in the control subject after stimulation of NK cells with IL-2 and IL-15 (Figure 8 C). Conversely, stimulation with IL-15 lead to a significantly lower

increase of IFNG and SOCS1 mRNAs expression in the cells from the patient as compared to healthy control (Figure 8, panels D and E).

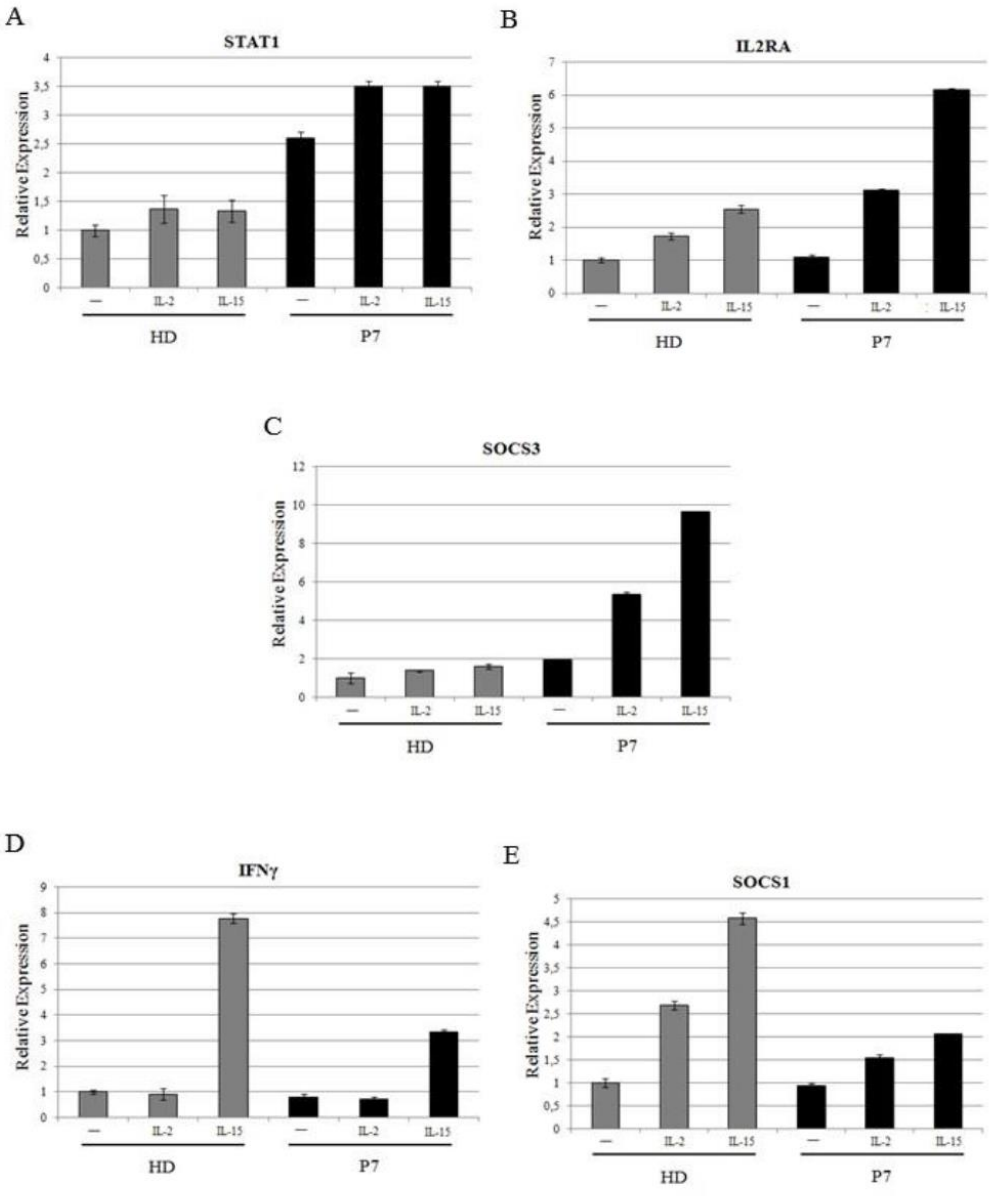

Figure 8. Abnormal gene response in NK cells of CMCD patients to IL-15. (A - E) IL-2-activated NK cells of patient P7 and a healthy subject (HD) were stimulated in vitro for 24

hours with IL-2 (100 ng/ml) or IL-15 (50 ng/ml) or were left unstimulated (-). The transcription of STAT1 (A), IL-2RA (B), SOCS3 (C), IFNγ and SOCS1 (E) was measured using RT-PCR. Target gene expression was normalized to the housekeeping gene (GAPDH) expression and presented as n-fold of the expression in unstimulated cells from the healthy control. The experiments shown are representative of three independent experiments. Data are presented as mean ± SE.

NK cell proliferation and IFN-γ production

Activated NK cells release cytokines that can modulate various effector functions of the immune system. A significantly reduced production of IFN-γ in response to IL-15 stimulation was reported in NK cells from CMCD patients as compared to healthy controls ($p<0.05$) (Figure 9 A, left panel). In contrast, IFN-γ production after IL-12 and IL-18 stimulations was normal, suggesting a selective defect of NK response to IL-15 stimulation (Figure 9 A, right panel).

The expression of the proliferation marker Ki67 on CD56+/CD3- cells after stimulation with IL-15 or IL-2 was significantly lower ($p< 0.05$) in cells from CMCD patients as compared to control NK cells (Figure 9 B). Moreover, Ki67 expression was considerably decreased in NK cell derived from patients P3, P7 and P8 after activation for 48h with IL-2 or IL-15, as compared to response of NK cells derived from a representative healthy donor (Figure 9 C). These results suggest that NK cells derived from CMCD patients display an impaired response to cytokines that signal through STAT5, such as IL-2 and IL-15.

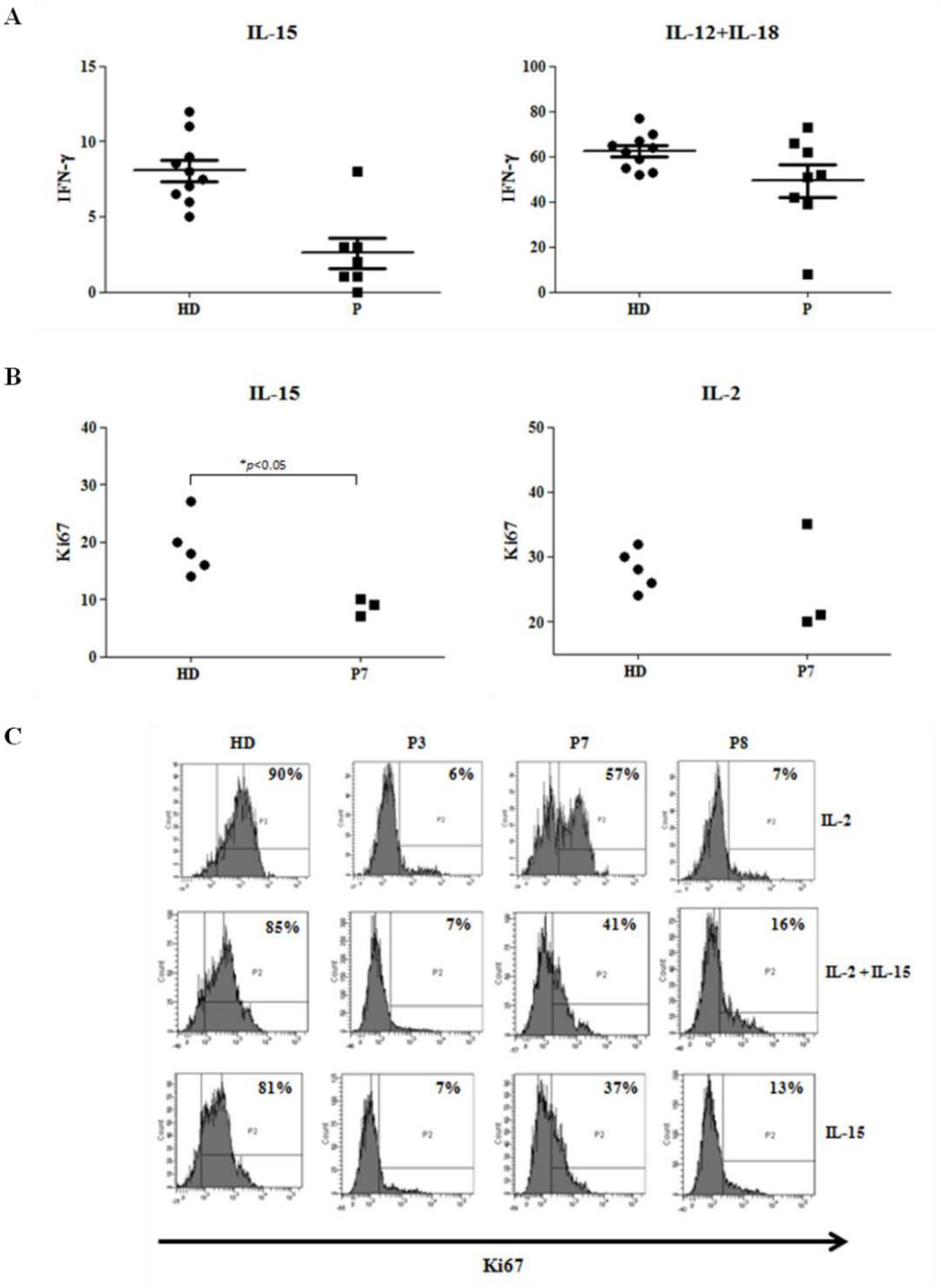

Figure 9. Reduced IFN-gamma production and Ki67 expression by NK cells in CMCD patients. (A) IFN-γ production was measured by intracellular staining from freshly isolated

CD56+CD3- NK cells derived from eight STAT1 patients (black square) and ten healthy donors (black circle) after overnight stimulation with IL-15 (A, left panel, $p<0.05$) or IL-12+IL-18 (A, right panel). (B) Ki67 expression by intracellular staining was measured in CD56+/CD3- NK cells in PBMC that were freshly isolated from three CMCD patients (square) and five healthy donors (circle) after stimulation for 72 hours with IL-15 (B, left panel, $p<0.05$) or IL-2 (B, right panel). (C) NK cells derived from three CMCD patients (P3, P7, P8) and one representative healthy donor were kept in culture with IL-2, maintained for 16 hours in complete medium without IL-2 and then stimulated with IL-2, IL-2 and IL-15, or IL-15 alone. After 48 hours, the expression of the intracytoplasmic protein Ki67 was analyzed by flow cytometry. The experiment we show herein is representative of three experiments that were performed.

Discussion

In CMCD patients STAT1-GOF mutations result in impaired generation of Th17 cells and increased risk of fungal and bacterial infections. In this study, we report that in CMCD increased rate of viral infections may be associated with abnormal NK cell functions and proliferation. Abnormal NK cells functioning have been previously reported in different PIDs such as Wiskott-Aldrich syndrome, DOCK8 deficiency or Hermansky-Pudlak type 2 syndrome (118-120). In all these conditions, the addition of IL-2 was able to correct the NK cytotoxicity defect, suggesting that the immunomodulatory activities and the response to IL-2 were preserved (120, 121).

In this study a reduced cytolytic activity against susceptible human erythroleukemia K562 cell line was reported IL-2-activated NK cells and, to lower extent, in unstimulated cells. This finding was not related to abnormal expression and/or functioning of NK receptors or to reduced perforin expression. Differently from what reported in other different PIDs, an impaired proliferative response to both IL-2 and IL-15, and an impaired IFN-γ production were observed in NK cells from CMCD patients. While IFN-γ release was normal in activated NK cells after stimulation with IL-12 or IL-18 (122).

Different soluble immune mediators, including IL-15 and IL-2, are able to modulate in vivo NK cells functions. IL-15 promotes survival of mature NK cells (123), controls NK homeostatic proliferation as well as proliferation following bacterial, viral, or fungal infections and synergizes with IL-12 and IL-18 to induce IFN-γ production by NK cells (122). IL-2 and IL-15 intracellular pathways share several signaling components, including the Janus Kinases (Jak) 1 and 3 molecules, bound to IL-15Rβ and common γc receptors and STAT5a and b transcription factors. Studies in mice and humans highlighted the importance of the STAT5 pathway for IL-15 biological activities on NK cells (124-126). A significant reduction of the extent of STAT5 phosphorylation in response to IL-2 or IL-15 was reported in NK cells from GOF-STAT1 patients, suggesting an impaired cellular response of NK cells to these cytokines. NK cells from such patients expressed normal levels of STAT5.

Interestingly, strong STAT1 phosphorylation after IL-15 or IL-2 stimulation was observed in NK cells from CMCD patients. In normal NK cells, signaling response to IL-2 or IL-15 can be detected only after prolonged exposure of the membrane. The increased

sensitivity of NK cells to STAT1-mediated signaling, observed in CMCD patients, might interfere with STAT5-dependent responses to IL-15 or IL-2. In conclusion, this evidence suggests that, persistent STAT1 phosphorylation, induced in response to various cytokines in different cell types, can interfere with important steps in the differentiation and functions of T and NK cells, resulting in impaired generation of Th17 cells and reduced proliferation of NK cells. The reduction of NK cell proliferative response associated to lower IFN-γ production in response to IL-15 or IL-2 might confer to CMCD patients an increased risk to develop invasive viral infections.

CHAPTER 3

"Ectodermal disorders and PIDs"

The description, in the last decades, of several novel forms of PIDs lead to discovery of new clinical and genetic aspects. Evidence shows that the skin represent the first defenses to hosts. In fact, it is the primary boundary for germs, and represent the main site of environment–host interactions. Recent studies suggest that it is also directly involved in the developmental process of the immune system. As a matter of fact, abnormalities of skin and skin annexa, leading to skin dryness, brittleness of hair, nail abnormalities and abnormal dentition, are often associated with different immunodeficiencies.

Ectodermal dysplasia (ED) is a group of rare (7 out of 10,000 live births) inherited disorders that involves two or more tissues of ectodermal origin. To date about 200 different forms of ectodermal dysplasia have been described. In patients affected with ED, skin is usually dry, thin and hypopigmented, and prone to rashes, eczema or infections. Sweat glands may also be involved and may function abnormally, or not develop at all. Abnormal sweating may alter the control of body temperature, leading to overheating, especially in hot environments. The involvement of airways seromucous glands may reduce the normal protective secretions of the mouth and nose, predisposing to recurrent respiratory infections. The involvement of meibomian/tarsal glands may cause dryness of the eye, cataracts, and vision defects. Hair is usually hypopigmented, thin and sparse. Teeth are usually congenitally absent, peg-shaped or pointed. The enamel may also be defective. Other clinical features include typical cranial-facial features (frontal bossing, longer or pronounced chins and broader noses) and abnormalities in the ear development leading to hearing problems.

The most common form is hypoidrotic ED (HED - incidence of 1:10,000 live birth) (127) which could be inherited as an autosomal dominant (AD), autosomal recessive (AR) or X-linked trait (XLHED). HED is caused by mutations in the ectodysplasin-A (EDA) signaling pathway, implicated in the development of the skin and skin appendage. Most of the cases of HED (approximately 80%) derives from mutations in the EDA gene on X-chromosome (OMIM 305100, XLHED, ectodermal dysplasia, type 1, ED1). A smaller subset derives from mutations in the EDA receptor (EDAR), the adapter protein (EDARADD), or WNT10A [2, 3], being inherited in an a.r. (ectodermal dysplasia anhidrotic; EDA; OMIM 224900) or AD manner (ectodermal dysplasia type 3; ED3;OMIM129490).

EDA plays a key role in organogenesis, from the initiation to the terminal differentiation (128, 129). Upon the binding of EDA ligand to the trimeric EDAR receptor, EDARADD adaptor is recruited, leading 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), is required for the activation of NF-kB pathway. IkB phosphorylation by NEMO, results in IkB degradation and NF-kB translocation into the nucleus (130).

Hypomorphic mutations in the IKBKG/NEMO gene on the X-chromosome result in HED with immunodeficiency (HED-ID,OMIM300291) (131-133). Differently from mutation in EDA pathway, mutations in IKBKG/NEMO gene are responsible for a heterogeneous and severe immunodeficiency. Even though recurrent bronchial or eye infections are described in patients with XLHED, in such patients recurrent infections do not result from PID but

develop as consequence of reduced bronchial or meibomian/tarsal gland function. In fact, the involvement of both NEMO and EDA-1 in the ectodysplasin pathway explains the similarity in the cutaneous involvement. However, NEMO is also involved in signaling pathways downstream to different receptors, including TLRs, interleukin-1 (IL-1Rs), tumor necrosis factor (TNFRs), and B- and T-cell receptors (TCR and BCR), thus explaining the wider immune defect in HED-ID and its complexity.

HED-ID patients suffer from recurrent, severe, and life-threatening bacterial infections. Infections usually involve lower respiratory tract, skin, soft tissues, bones and gastrointestinal apparatus. Meningitis and septicemia may develop in early childhood. Infections by Gram-positive bacteria (S. pneumoniae and S. aureus), followed by Gram-negative bacteria (Pseudomonas spp. and Haemophilus influenzae) and mycobacteria usually feature this syndrome. Patients usually show hypogammaglobulinaemia with low serum IgG (or IgG2) levels, and variable levels of other immunoglobulin isotypes (IgA, IgM and IgE). Elevated serum IgM levels may also be observed, featuring the hyper-IgM6 phenotype (134, 135). The hyper-IgM phenotype may derive from a defective ability of B cells to switch in response to CD40 ligand (CD40L).

Antibody response to polysaccharide and proteic antigens is also impaired. Alterations of NK activity have also been reported in some (136, 137) but not all (136, 137) patients with EDA-ID. Poor inflammatory response, due to impaired cellular responses to TLR and pro-inflammatory cytokines (IL-1β, IL-18andTNF-α) (138) are often reported, thus explaining the occurrence of severe mycobacterial disease in these patients.

Different NEMO mutations have been associated with distinct disorders. Loss-of-function mutations cause incontinentia pigmenti (IP), which is lethal in males nad specifically affects females, being (139). 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 (OLHED-ID; MIM 300301).

Mutations in other genes involved in NF-κB pathway are responsible for different forms of HED-ID. Gain-of-function mutations of IκBα are able to enhance the inhibitory capacity of IκBα through the prevention of its phosphorylation and degradation, and result in impaired NF-κB activation. The developmental, immunologic and infectious phenotypes associated with hypomorphic NEMO and hypermorphic IKBA mutations largely overlap and include EDA, impaired cellular responses to ligands of TIR (TLR-ligands, IL-1β and IL-18), and TNFR (TNF-α,LTα1/β2 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 CVID, inherited as an autosomal dominant trait. In the cases so far identified, ectodermal abnormalities, including nail dystrophy and alopecia together with endocrine alterations, have been reported.

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

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 (140, 141). Immunodeficiency results in susceptibility to encapsulated pyogenic bacteria, such as Streptococcus pneumoniae and Haemophilus influenzae, mycobacteria, and herpes virus infections (142, 143). The involvement of NEMO in different cellular pathways helps explaining the complexity of the immunological phenotype, However, to date, little is known about the pathogenesis of the humoral defects. In fact, humoral alterations may be explained only in part by the involvement of NF-kB in the CD40 signaling pathway. In fact, evidence shows that, in a large cohort of patients, in which hypogammaglobulinemia occurred in 59% of the patients, impaired CD40 signaling was reported only in patients carrying Zinc Finger. Recent studies show that there is a relationship between T-independent B-cell immunity and the response against polysaccharides of encapsulated bacteria (144). In fact, patients at higher risk of recurrent infections caused by encapsulated bacteria usually show alterations of IgM memory B cells, of T-independent B cell differentiation and of the ability to generate anti-pneumococcal polysaccharide IgM (145, 146), similarly to what happens in splenectomized or asplenic patients (144). TLR9 signaling is required for a proper differentiation of transitional B cells into IgM memory B cells (147). Defect of other molecules implicated in Toll–IL-1R (TIR) signaling pathway, acting upstream NEMO, such as IRAK-4 and Myd88

deficiencies, have been associated to increased susceptibility to invasive bacterial infections caused by S. pneumoniae (148). Moreover there is evidence that deficiencies 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 may be found in patients affected with IRAK-4 and Myd88 (149).

Conclusive remarks

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 published as *Article* on *Clinical Immunology* 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.

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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#-CCCT TGCCCTGTTGGATGAATAGGC-3#; reverse, 5#-AGGCGGGAGAGGAAAG CGCAGACTG-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#-CCCT ACCAAGAAGGTAGTTC-3#; forward, 5#-GATCCCTCCTAGTGACTATC-3#; reverse, 5#-CAGACAGACAATGCTGAAAGA-3#; forward, 5#-AAAA AAGTAACACTGAATCCTATT-3#; reverse 5#-CTCTCAGGATCACCCACTC-3#; forward, 5#-GGAAGTCAAAAGATTATGCCC-3#; reverse, 5#-CTAC CCAGGAAGAGAGCAAT-3#; forward, 5#-CTGAGCAAGCAGCCATTACT-3#; reverse, GGGGAGAAGCTCCTCTTTG-3#; forward, 5#-ACTGAGTGAC TGCCTTCTCT-3#; reverse, 5#-GCACCGGATCTGCATTCTGG-3#; forward, 5#-TGTCAATTCACCACAGGGAG-3#; reverse, 5#-CACAGCAGCACTTAGA GG-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].

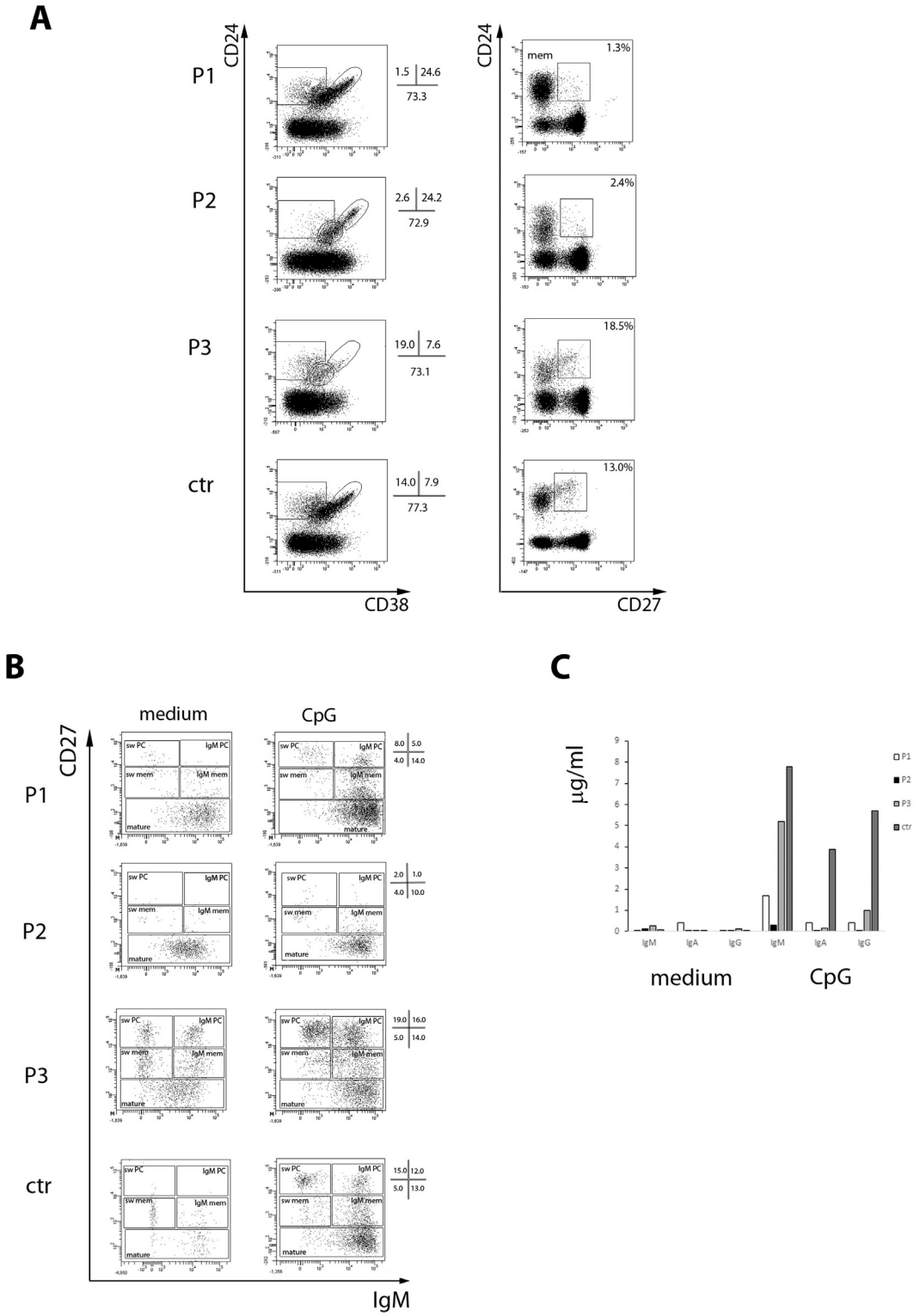

Fig. 1. B-cell phenotype and function of HED-ID and XLHED patients. (a)The left panel shows the identification of memory (CD3−CD19+CD24+CD27+), transitional (CD3−CD19+CD24+CD38hiCD27−), and mature B cells (CD3−CD19+CD24−CD38dim/loCD27−) in CD3−CD19+ gated cells using the CD24 and CD38 markers. In the 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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3.2 Insight into IKBKG/NEMO locus: report of new mutations and complex genomic rearrangements leading to incontinentia pigmenti disease.

Incontinentia pigmenti (IP; MIM #308300) is an X-linked disorder of the skin. The inheritance pattern is X-linked dominant, being lethal in males. Skin lesions evolve through four stages: a vesicobullous, verrucous, hyperpigmented, and finally a hypopigmented stage (150, 151). Apart from skin, also other different tissues of ectodermal origin including teeth, hair and nails, eyes, and central nervous system (CNS) may be affected. The severity of the disease depends on the extension of neurological and/or ocular involvement. Neurological manifestations may range from a single-seizure episode to severe motor and intellectual disability. Ocular involvement may lead to strabismus, retinopathy, congenital cataract, and microphthalmia (152-154). IP derives from mutations of IKBKG/NEMO gene (155, 156), which are in most of the cases loss-of-function (LoF), although also hypomorphic mutations have been reported (153, 156). NEMO/IKKgamma, the product of IKBKG/NEMO, is required for canonical NF-kB pathway activation (157, 158). The large heterogeneity of defects and the severe clinical presentations of IP might be due the pleiotropic role of NF-kB pathway (158). Hypomorphic IKBKG/NEMO mutations in male patients lead to EDA-ID, in which cutaneous involvement is associated with a severe and life-threatening Immune Deficiency (EDA-ID; MIM #300291) (159-162). EDA-ID and IP have completely different clinical presentations.

In this update, accepted for the publication as *Article* on *Human Mutation* we present 21 previously unreported IKBKG/NEMO mutations (nine small indels, and 12 SNPs) found in

IP patients. All variants, including relevant clinical data, were entered into the new IP locus-specific database at http://IKBKG.lovd.nl.

Conclusive remarks

The IKBKG/NEMO locus predispose to genomic instability leading to high frequency of de novo complex rearrangements observed in IP patients. The analysis of the effect of IKBKG/NEMO IP-associated missense mutations on the NF-kB signaling may help understanding the patho-physiological aspects of this rare NF-kB-related disease.

MUTATION UPDATE

Human Mutation

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Insight into *IKBKG/NEMO* Locus: Report of New Mutations and Complex Genomic Rearrangements Leading to Incontinentia Pigmenti Disease

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ABSTRACT: Incontinentia pigmenti (IP) is an X-linked-dominant Mendelian disorder caused by mutation in the *IKBKG/NEMO* gene, encoding for NEMO/IKKgamma, a regulatory protein of nuclear factor kappaB (NF-kB) signaling. In more than 80% of cases, IP is due to recurrent or nonrecurrent deletions causing loss-of-function (LoF) of NEMO/IKKgamma. We review how the local architecture of the *IKBKG/NEMO* locus with segmental duplication and a high frequency of repetitive elements favor de novo aberrant recombination through different mechanisms producing genomic microdeletion. We report here a new microindel (c.436_471delinsT, p.Val146X) arising through a DNA-replication-repair fork-stalling-and-template-switching and microhomology-mediated-end-joining mechanism in a sporadic IP case. The LoF mutations of *IKBKG/NEMO* leading to IP include small insertions/deletions (indel) causing frameshift and premature stop codons, which account for 10% of cases. We here present 21 point mutations previously unreported, which further extend the spectrum of pathologic variants: 14/21 predict LoF because of premature stop codon (6/14) or frameshift (8/14), whereas 7/21 predict a partial loss of NEMO/IKKgamma activity (two splicing and five missense). We review how the analysis of IP-associated *IKBKG/NEMO* hypomorphic mutants has contributed to the understanding of the pathophysiological mechanism of IP disease and has provided important information on affected NF-kB signaling. We built a locus-specific database listing all *IKBKG/NEMO* variants, accessible at http://IKBKG.lovd.nl.

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KEY WORDS: Incontinentia Pigmenti; *IKBKG*; *NEMO*; genomic rearrangements; NF-kB pathway

Introduction

Incontinentia pigmenti (IP; MIM #308300) is an X-linked multisystem disorder, affecting the skin in all patients, but also other ectodermal tissues comprising teeth, hair and nails, eyes, and central nervous system (CNS). Skin lesions are highly diagnostic, from the initial manifestations in neonates followed by the typical evolution through four stages over months to years, namely, a vesicobullous stage, verrucous stage, hyperpigmented stage, and finally a hypopigmented stage usually lasting a lifetime [Landy and Donnai, 1993; Scheuerle and Ursini, 2010]. The severity of the disease is related to neurological and/or ocular impairment. Overall, the prevalence of functional CNS manifestations is approximately 30% [Fusco et al., 2004; Meuwissen and Mancini, 2012; Minić et al., 2013a] ranging from a single-seizure episode to severe motor and intellectual disability. Ophthalmologic abnormalities comprising strabismus, retinopathy, congenital cataract, and microphthalmia are present in approximately 20%–37% of IP patients [Hadj-Rabia et al., 2003; Fusco et al., 2004; Minić et al., 2013a].

The inheritance pattern is X-linked dominant, with usually male lethality. However, some affected males have been reported; few of them present X chromosome somatic mosaicism, and in others a concomitant diagnosis of Klinefelter syndrome was demonstrated [Scheuerle, 1998; Aradhya et al., 2001b; Kenwrick et al., 2001; Fusco et al., 2007].

Additional Supporting Information may be found in the online version of this article.

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Inhibitor of Kappa light polypeptide gene enhancer in B-cells, Kinase Gamma/Nuclear factor kappaB, Essential MOdulator (*IKBKG/NEMO*) gene (HGNC-approved symbol *IKBKG*; GenBank NM_003639.3, MIM #300248), mutations are the cause of IP [Smahi et al., 2000; Fusco et al., 2008]. In most patients, the disease is due to loss-of-function (LoF) mutations, although hypomorphic mutations have been reported [Fusco et al., 2004, 2008]. IP is the first inherited disease affecting one of the core components of nuclear factor kappaB (NF-kB) signaling [Courtois and Gilmore, 2006]. The *IKBKG*/*NEMO* encodes for NEMO/IKKgamma a regulatory subunit of the inhibitor of the kappaB (IkB) kinase (IKK) complex required for canonical NF-kB pathway activation involved in many fundamental physiological and pathologic functions including cell survival [Hayden and Ghosh, 2004; Nelson, 2006]. In fact, the absence of NEMO/IKKgamma protein makes the cells sensitive to apoptosis, which produces skewed X inactivation in females [Fusco et al., 2004], whereas in males, extensive apoptosis is responsible for early foetal lethality [Courtois et al., 2001]. The large heterogeneity of defects and the severe clinical presentations of IP might be due to an X-inactivation in heterozygous IP females, coupled with the pleiotropic role of NF-kB pathway that is always affected in this pathology [Nelson, 2006].

The second for frequency inherited disease due to *IKBKG/NEMO* mutations is Ectodermal Dysplasia, Anhidrotic, associated with a severe and life-threatening Immune Deficiency (EDA-ID; MIM #300291) in males [Aradhya et al., 2001b]. In EDA-ID male patients, the disease is always associated with hypomorphic *IKBKG*/*NEMO* mutations, because the absence of gene produces male lethality during embryogenesis [Zonana et al., 2000; Döffinger et al., 2001; Dupuis-Girod et al., 2002]. EDA-ID and IP have completely different clinical presentations.

Since the discovery that *IKBKG*/*NEMO* mutations cause IP, it has been increasingly clear that most of alterations are the consequence of the local genomic instability due to structural architecture in which the gene is located [Aradhya et al., 2001a, 2001c; Fusco et al., 2009, 2012a]. Indeed, *IKBKG/NEMO* partially overlaps, on the 5′-side, with *Glucose-6-Phosphate Dehydrogenase* (*G6PD*, GenBank NM_000402) gene and on the 3′-side, with low-copy-repeat (LCR1) (Fig. 1A). In addition, in the same locus, a nonfunctional, highly conserved pseudogene copy (*IKBKGP*/*NEMOP)* maps in the LCR2 (the inverted paralogous sequence of LCR1) and is involved in rare inversion/gene-conversion events causing the repositioning/copying of mutations from pseudogene to gene [Fusco et al., 2012a].

Moreover, the presence of multiple SINEs (Short INterspersed Elements) and LINEs (Long INterspersed Elements), micro-/macro-homologies, and tandem repeats in both LCRs increases genomic instability of the locus promoting aberrant recombinations that account for both recurrent and nonrecurrent deletions (Supp. Fig. S1).

Although a recurrent deletion (*IKBKGdel*), removing the exon 4–10 and causing a LoF of *IKBKG/NEMO* is frequently associated with IP (78% of cases), the molecular diagnosis of heterozygous females is quite complicated because of the *IKBKGP*/*NEMOP* and also because mutations are scattered all along the gene [Fusco et al., 2012b]. However, we and others have expanded the catalog of different alterations that could affect *IKBKG*/*NEMO* [Fusco et al., 2009, 2012a; Hsiao et al., 2010; Fryssira et al., 2011]. In this update, we present 21 previously unreported *IKBKG*/*NEMO* mutations (nine small indels, and 12 single-nucleotide substitutions) found in IP patients including a new indel mutation (c.436_471delinsT) that alters the open reading frame of NEMO/IKKgamma by a premature stop codon (p.Val146X). Moreover, we will provide an overview of all *IKBKG*/*NEMO* submicroscopic genomic rearrangements found in IP patients in the recent years, integrated with an extensive description of different genomic mechanisms likely involved in their generation, including the "DNA-replication/repair-based" mechanism that probably has generated the de novo c.436_471delinsT mutation, here reported. All variants, including relevant clinical data, were entered into the new IP locus-specific database at http://IKBKG.lovd.nl.

Taken together, past and present data demonstrate that the architecture of the *IKBKG*/*NEMO* locus facilitates genomic instability generating the high frequency of de novo complex rearrangements observed in IP patients. Moreover, we illustrate how, in recent years, the analysis of the effect of *IKBKG*/*NEMO* IP-associated missense mutations on the NF-kB signaling has contributed to understanding the patho-physiological aspects of this rare NF-kB-related disease.

The *IKBKG* Mutation Database

We have established a Web-based IP-specific locus-specific database that gives a complete overview of the variants identified in *IKBKG*, and we have transferred to the Web-accessible all available information on *IKBKG* mutations and their IP carriers. This database is included in the Human Genome Variation Society (www.HGVS.org) list of locus-specific databases. The database contains all the *IKBKG* mutations, unclassified variants, and benign variants, which have been published in the medical literature, including those presented in this article. It is IP-patient-based and mutation-based database, because it contains information about the clinical phenotype of the patient (if provided) and about the effect of pathogenicity of the mutation (if defined). Indeed, this *IKBKG* mutation database has been constructed to aid both clinicians and scientists.

The database will be freely accessible online at http://IKBKG.lovd.nl. It can be updated with any reported variant from any team, worldwide. It is highly recommended that new as well as previously reported variants are submitted to the database because additional data will improve its value, for example, for the interpretation of unclassified variants and phenotype—genotype correlations.

Summary of Clinical Findings in Unpublished Patients

Fifty-three IP patients (14.8%) with point mutations (21 previously unreported mutations and 32 reported mutations) in the *IKBKG* gene were identified in 358 IP patients analyzed by the CNR-IGB and Necker Hospital Laboratories between 2008 and 2013. In this cohort, the 72% (258/358 IP patients) carried the recurrent deletion (*IKBKGdel*), whereas the 13% (47/358 IP patients) did not show any alteration in *IKBKG* gene. The patients were referred from other European countries for analysis, and their clinical information was collected by using a clinical extensive questionnaire (approved by The International IP Consortium, www.ipif.org) reporting all aspects of IP phenotype: skin, hair, teeth, nails, eyes, and CNS defects. All the IP patients analyzed in this study met the 1993 revised criteria for the classification of IP [Landy and Donnai, 1993; Scheuerle and Ursini, 2010; Minić et al., 2013b].

All affected females underwent skin biopsy obtained from the limbs, usually from the legs. The cutaneous lesions (erythema, vesicles, and pustules in the first stage, followed by verrucous and keratotic lesions in the second stage, linear hyperpigmentation in the third stage and pale, hairless, scarring patches in the fourth

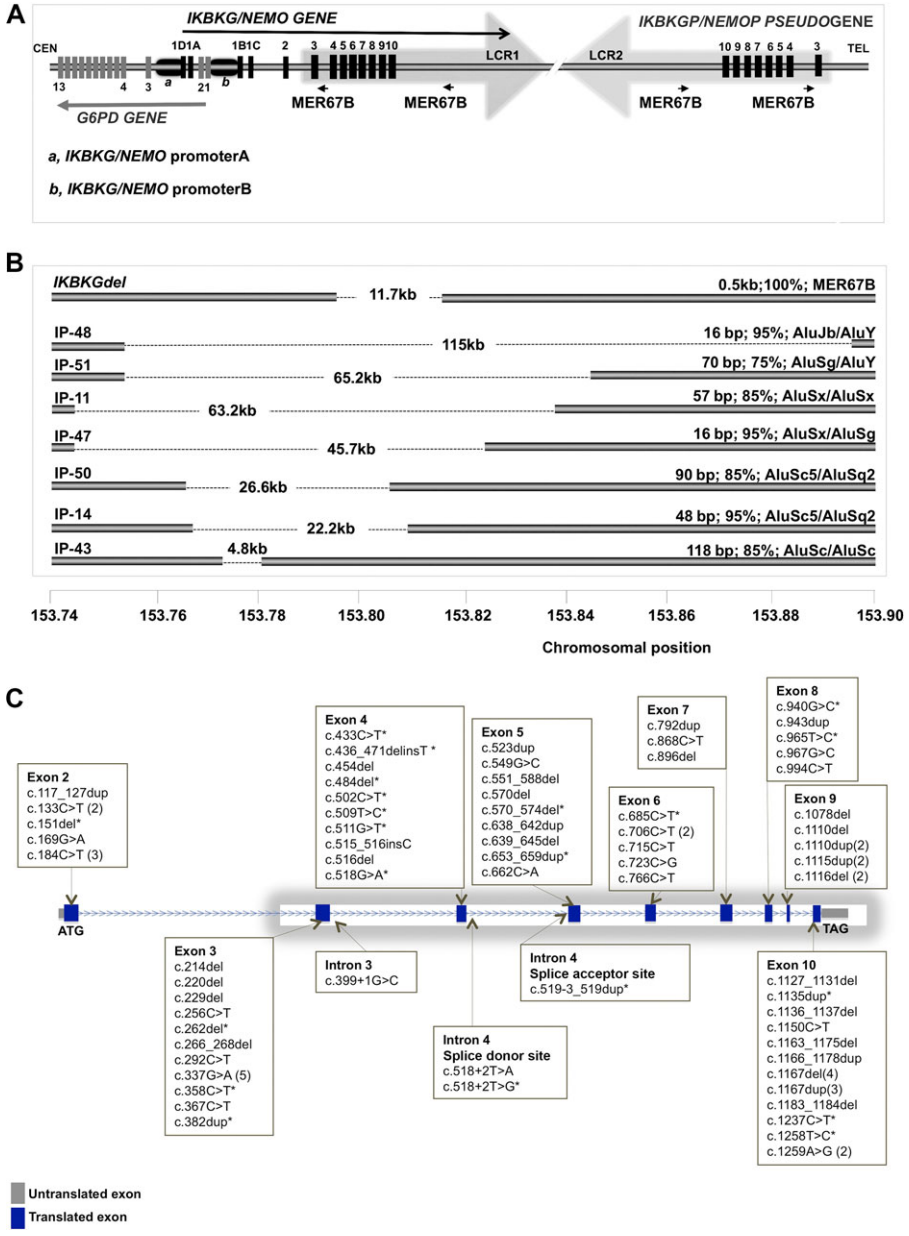

Figure 1. **A:** Genomic structure of the IP locus in Xq28. The square arrows represent LCRs (LCR1 and LCR2). *G6PD* exons (light gray boxes), *IKBKG* exons (black boxes), *IKBKGP* exons (dark gray boxes). The exons are numbered for each gene. The long arrows indicate the transcriptional direction for each gene, whereas the small arrows indicate the MER67B repeated sequences. **B:** Overview of the recurrent and nonrecurrent rearrangements at the IP locus. The location and size (kb) of each deletion is illustrated by horizontal dotted line. Microhomology sequences (extention_bp and percentage of homology) and repeat sequences used as substrates in each rearrangement are reported. In IP-43, a NAHR event between two *AluSx* repeats has caused the deletion of genomic fragment because two defined homology regions with 86% sequence identity, enriched by two long tandem repeats and by a high density of *Alu* repeated sequences, were mapped at the breakpoint junctions. **C:** Schematic representation of the coding region of *IKBKG* gene (from exon 2 to exon 10). The position of mutations (as reported in Table 1) causing IP disease is shown in exon/intron box. The symbol (*) indicates the new mutations reported in this paper, whereas numbers in brackets indicate how often the recurrent mutations were reported. The shaded rectangle represents the duplicated region (LCR1).

stage) were observed in all patients (Table 1). The other major clinical findings associated with mutations are provided in Table 1. Data relating to the onset of IP were available for 10 patients.

IP Sporadic Cases

The IP syndrome is typically a sporadic condition, familial recurrence is rare, and most of *IKBKG* mutations occur de novo (65% of cases) [Smahi et al., 2000; Aradhya et al., 2001c; Fusco et al., 2004; see also Nelson, 2006].

Thirteen de novo mutations reported in the literature (Table 2) and seven of the new cases reported here (Table 1) are confirmed to be de novo.

Mutation Spectrum in IP Locus

Point Variations in the *IKBKG* Gene

Mutations are named according to HGVS nomenclature guidelines (www.HGVS.org), numbered with respect to the *IKBKG*

Table 1. Genetic and Clinical Summary of novel *IKBKG/NEMO* Mutations Reported in IP Patients

IP	Nucleotide change NM_003639.3	Protein change NP_003630.1	Exon	Mutation type	Inheritance	XIC	CNS defects	Ocular defects	Dental defects	Hair defects	Nail defects
1	c.151del	p.Leu51SerfsX64	Exon 2	Frameshift	nd	nd	–	Present	Present	Present	–
2	c.262del	p.Glu88ArgfsX27	Exon 3	Frameshift	S	SK	–	Present	Present	Present	Present
3	c.358C>T	p.Gln120X	Exon 3	Nonsense	F	SK	–	–	–	Present	–
4	c.382dup	p.Leu128ProfsX10	Exon 3	Frameshift	nd	SK	–	Present	Present	Present	–
5	c.433C>T	p.Gln145X	Exon 4	Nonsense	S	SK	–	Present	Present	–	–
6	c.436_471delinsT	p.Val146X	Exon 4	Nonsense	nd	SK	Present	–	Present	–	–
7	c.484del	p.Ala162LeufsX119	Exon 4	Frameshift	S	SK	–	–	Present	–	–
8	c.502C>T	p.Gln168X	Exon 4	Nonsense	nd	SK	–	–	Present	–	Present
9	c.509T>C	p.Leu170Pro	Exon 4	Missense	F	SK	–	–	–	–	–
10	c.511G>T	p.Glu171X	Exon 4	Nonsense	S	–	–	–	Present	–	–
11	c.518G>A[a]	p.Arg173Gln	Exon 4	Missense	nd	SK	–	–	Present	–	–
12	c.518+2T>G	nd	Intron 4	Splice site	nd	nd	–	Present	Present	–	–
13	c.519–3_519dup	nd	Intron 4	Splice site	nd	R	Present	–	Present	Present	–
14	c.570_574del	p.Leu191AlafsX61	Exon 5	Frameshift	nd	SK	–	–	Present	–	–
15	c.653_659dup	p.Ser221GlyfsX35	Exon 5	Frameshift	nd	SK	–	Present	Present	–	–
16	c.685C>T	p.Gln229X	Exon 6	Nonsense	S	SK	–	–	Present	Present	–
17	c.940G>C	p.Ala314Pro	Exon 8	Missense	S	R	–	–	Present	Present	–
18	c.965T>C	p.Leu322Pro	Exon 8	Missense	S	SK	–	–	–	–	–
19	c.1135dup	p.Leu379ProfsX16	Exon 10	Frameshift	F	SK	Present	–	Present	–	–
20	c.1237C>T	p.His413Tyr	Exon 10	Missense	nd	R	–	–	Present	Present	–
21	c.1258T>C	p.X420GlnextX27	Exon 10	Frameshift	nd	R	–	–	Present	–	–

[a]This mutation is predicted to alter also the splicing-site exon 4/intron 4.
Skin defects are present in all patients.
Present/–, presence/absence of complications for each tissue.
CNS defects (seizures, spastic paresis, motor retardation, mental retardation, or microcephaly); ocular defects (strabismus, cataracts, optic atrophy, retinal vascular pigmentary abnormalities, microphtalmous, or pseudogliomas); dental system defects (partial anodontia, delayed dentition, cone/peg shaped teeth, or impactions); hair defects (vertex alopecia, wooly hair naevus, or eyelash and eyebrow hypogenesis); and nail defects (onychogryphosis, pitting or ridging).
nd, not detected; XIC, X-inactivation chromosome test on lymphocytes of the patient [Allen et al., 1992]; SK, skewed, R, random, X-inactivation profile. S, sporadic case; F, familial case.

gene reference sequence (AJ271718.1 covering *IKBKG* gene transcript NM_003639.3), and nomenclature of all mutations have been checked using Mutalyzer (www.LOVD.nl/mutalyzer/) [Wildeman et al., 2008]. All base changes are predicted to be pathogenic based on their location in functional domains.

Forty-eight unique point mutations or small indels have been reported previously in the literature (Table 2) and this paper adds other 21 (Table 1) to the list of mutations identified in IP patients (69 IP mutations). Most reported mutations, inclusive of the data, are indel mutations 54% (37/69), whereas the 46% (32/69) are single-nucleotide substitution. Moreover, the effects predicted on the mutated protein show that 52% (36/69) cause a frameshift, the 26% (18/69) a premature stop codon, the 15% (10/69) are missense mutations, the 6% (4/69) are splice-site mutations, and only 1% (1/69) is an in-frame amino acid deletion (p.Lys90del, Table 2).

The majority of mutations have only been reported once, and only 10 mutations have been reported at least twice (Fig. 1C). The variants p.Glu390ArgfsX5 (c.1167dup, exon10) and p.Glu390ArgfsX61 (c.1167del, exon10) have been reported three and four times, respectively (Fig. 1C) [Zonana et al., 2000; Aradhya et al., 2001b; Döffinger et al., 2001; Lee et al., 2005; Pachlopnik Schmid et al., 2006; Tono et al., 2007; Chang et al., 2008; Fusco et al., 2008; Mancini et al., 2008]. Both mutations are located in the same stretch of cytosine of exon 10 that is prone to generate errors during the DNA replication by slippage mechanism [Fusco et al., 2008]. The p.Asp113Asn (c.337G>A, exon3) was described as a polymorphic variant of the *IKBKG* gene in Fusco et al. (2004) and, more recently, in Tarpey et al. (2009). Moreover, in Salt et al. (2008), it was reported in unaffected female and in her son associated with selective susceptibility to *Pneumocystis* and *Cytomegalovirus* infections without any signs of ectodermal dysplasia. These observations exclude any correlation of this *IKBKG* variant with IP. Mutations in regulatory domains or intronic regions outside the sequence immediately flanking the exons have not been described, most likely because these regions of the gene are not routinely screened or have yet to be fully characterized.

Splice-site mutations

To date, only two splice-site mutations have been reported in IP (Table 2). They are a single-nucleotide substitution at the splice donor site, c.399+1G>C (intron3) and c.518+2T>A (intron4).

We report here two new splice-site mutations, both in intron4, c.518+2T>G (splice donor site) and c.519-3_519dup (splice acceptor site) (Fig. 1C, Table 1). We predicted the effect on the splicing by NetGene software (http://www.cbs.dtu.dk/services/NetGene2/) and we observed that aberrant transcripts would be generated.

Moreover, we found in IP patient (IP-11 in Table 1) a previously unreported mutation c.518G>A due to a single-nucleotide substitution in the last position of *IKBKG* exon 4 nucleotide. The effects that we could predict are two: a missense mutation (p.Arg173Gln, as reported in Table 1) and an abnormal transcript because the splice donor site is affected.

The predicted sequence of mutated proteins results in a premature termination that causes the LoF of the NEMO/IKKgamma. It might be also speculated that aberrant splicing products may have an accelerated degradation of mutated mRNA by the mechanism of nonsense-mediated decay [Baker and Parker, 2004].

Large Variations in the *IKBKG* Gene

IKBKG comprises nine coding exons (exons 2–10) and four alternative noncoding first exons (Fig. 1A). Transcription from exons 1B and 1C is directed by the strong bidirectional promoter B (*b* in Fig. 1A), in the CpG island, whereas transcription from exons 1D and 1A is directed by a weak unidirectional promoter A (*a* in

Table 2. *IKBKG/NEMO* Mutations Reported IP Patients

IP	Nucleotide change NM_003639.3	Amino acid change NP_003630.1	Exon	Mutation type	Inheritance	Clinical diagnosis	References
1	c.117_127dup	p.Ser43CysfsX76	Exon 2	Frameshift	F	IP	[Smahi et al., 2000]
2	c.133C>T	p.Gln45X	Exon 2	Nonsense	F	IP	[Fusco et al., 2008]
3	c.169G>A	p.Glu57Lys	Exon 2	Missense	F	IP	[Aradhya et al., 2001c]
4	c.184C >T	p.Arg62 X	Exon 2	Nonsense	F	IP	[Fusco et al., 2008; Smahi et al., 2000]
5	c.214del	p.Leu72Cys_fs X 43	Exon 3	Frameshift	ND	IP	[Aradhya et al., 2001c]
6	c.220del	p.Glu74Ser_fsX41	Exon 3	Frameshift	ND	IP	[Aradhya et al., 2001c]
7	c.229del	p.Glu77Arg_fs X 38	Exon 3	Frameshift	S	IP	[Fusco et al., 2008]
8	c.256C>T	p.Gln86 X	Exon 3	Nonsense	F	IP	[Fusco et al., 2008]
9	c.266_268del	p.Lys90del	Exon 3	InFramedel	S	IP	[Fusco et al., 2004]
10	c.292C>T	p.Gln98 X	Exon 3	Nonsense	ND	IP	[Aradhya et al., 2001c]
11	c.337G>A	p.Asp113Asn	Exon 3	Missense	S	IP	[Fusco et al., 2004, 2008]
12	c.367C>T	p.Arg123Trp	Exon 3	Missense	F	IP	[Fusco et al., 2004]
13	c.399+1G>C	ND	Intron 3	Splice site	ND	IP	[Aradhya et al., 2001c]
14	c.454del	p.Glu152SerfsX129	Exon 4	Frameshift	F	IP	[Fusco et al., 2008]
15	c.515_516insC	p.Arg173SerfsX15	Exon 4	Frameshift	F	IP	[Fusco et al., 2008]
16	c.516del	p.Arg173GlyfsX108	Exon 4	Frameshift	ND	IP	[Aradhya et al., 2001c]
17	c.518+2T>A	ND	Intron 4	Splice site	ND	IP	[Aradhya et al., 2001c]
18	c.523dup	p.Arg175ProfsX13	Exon 5	Frameshift	ND	IP	[Aradhya et al., 2001c]
19	c.G549C	p.Gln183His	Exon 5	Missense	S	IP	[Hsiao et al., 2010]
20	c.551_588del	p.Leu184ArgfsX57	Exon 5	Frameshift	ND	IP	[Aradhya et al., 2001c]
21	c.570del	p.Leu191CysfsX90	Exon 5	Frameshift	ND	IP	[Aradhya et al., 2001c]
22	c.638_642dup	p.Met215SerfsX68	Exon 5	Frameshift	ND	IP	[Aradhya et al., 2001c]
23	c.639_645del	p.Arg214SerfsX65	Exon 5	Frameshift	ND	IP	[Aradhya et al., 2001c]
24	c.662C>A	p.Ser221X	Exon 5	Nonsense	ND	IP	[Fusco et al., 2008]
25	c.706C>T	p.Gln236X	Exon 6	Nonsense	S	IP	[Aradhya et al., 2001c]
26	c.715C>T	p.Gln239X	Exon 6	Nonsense	S	IP	[Fusco et al., 2004]
27	c.723C>G	p.Tyr241X	Exon 6	Nonsense	ND	IP	[Aradhya et al., 2001c]
28	c.766C>T	p.Arg256X	Exon 6	Nonsense	F	IP	[Fusco et al., 2008]
29	c.792dup	p.Gln265ThrfsX19	Exon 7	Frameshift	S	IP	[Martinez-Pomar et al., 2005].
30	c.868C>T	p.Gln290 X	Exon 7	Nonsense	ND	IP	[Aradhya et al., 2001c]
31	c.896del	p.Pro299ArgfsX3	Exon 7	Frameshift	ND	IP	[Aradhya et al., 2001c]
32	c.943dup	p.Glu315GlyfsX80	Exon 8	Frameshift	F	IP	[Fusco et al., 2004]
33	c.967G>C	p.Ala323Pro	Exon 8	Missense	S	IP	[Sebban-Benin and Pescatore et al., 2007]
34	c.994C>T	p.Gln332X	Exon 8	Nonsense	S	IP	[Fryssira et al., 2011]
35	c.1078del	p.His360MetfsX91	Exon 9	Frameshift	S	IP	[Fusco et al., 2004]
36	c.1110del	p.Ala371ProfsX80	Exon 9	Frameshift	ND	IP	[Fusco et al., 2008]
37	c.1110dup	p.Ala371ArgfsX24	Exon 9	Frameshift	F	IP; EDA-ID	[Smahi et al., 2000]
38	c.1115dup	p.Ala373CysfsX22	Exon 9	Frameshift	F	IP	[Fusco et al., 2008]
39	c.1116del	p.Ala373ProfsX78	Exon 9	Frameshift	S	IP	[Fusco et al., 2004]
40	c.1127_1131del	p.Ser377ProfsX16	Exon 10	Frameshift	F	IP	[Fusco et al., 2008]
41	c.1136_1137del	p.Leu379ArgfsX15	Exon 10	Frameshift	S	IP	[Fusco et al., 2008]
42	c.1150C>T	p.Gln384 X	Exon 10	Nonsense	S	IP	[Fusco et al., 2004]
43	c.1163_1175del	p.Pro388HisfsX59	Exon 10	Frameshift	F	IP	[Aradhya et al., 2001b, 2001c].
44	c.1166_1178dup	p.Asp394ArgfsX5	Exon 10	Frameshift	F	IP	[Aradhya et al., 2001b]
45	c.1167del	p.Glu390ArgfsX61	Exon 10	Frameshift	F	IP	[Aradhya et al., 2001b, 2001c; Fusco et al., 2008]
46	c.1167dup	p.Glu390Argfs X5	Exon 10	Frameshift	F	IP; EDA-ID	[Zonana et al., 2000]
47	c.1183_1184del	p.Phe395LeufsX11	Exon 10	Frameshift	F	IP; EDA-ID	[Fusco et al., 2008]
48	c.1259A>G	p.X420TrpextX27	Exon 10	Nonstop	F	IP; OL-EDA-ID	[Smahi et al., 2000]

ND, not detected; S, sporadic case; F, familial case.

Fig. 1A), in intron 2 of *G6PD* [Fusco et al., 2006]. The *IKBKG* gene, located in LCR1, and the nonfunctional *IKBKGP/NEMOP*, in LCR2 (Fig. 1A), share >99% identity and the presence of multiple repeat sequences (SINEs, LINEs, and LTRs; Supp. Fig. S1), micro-/macro-homologies and tandem repeats, make the IP locus highly vulnerable to damage, generating aberrant IP locus alleles named recurrent and/or nonrecurrent rearrangements, by different molecular mechanisms (nonallelic-homologous-recombination [NAHR], nonhomologous-end-joining [NHEJ], fork-stalling-and-template-switching [FoSTeS], and microhomology-mediated-end-joining [MMEJ]) [Fusco et al., 2012a].

Recurrent rearrangements in IP locus

The most frequent pathologic variant in IP is a deletion (*IKBKGdel*, 78% of IP cases, Fig. 1B), spanning exons 4–10 in the *IKBKG* gene [Smahi et al., 2000; Aradhya et al., 2001c; Fusco et al., 2004, 2008]. The deletion removes the region of ∼11.7 kb, between two MEdium Reiterated 67B (MER67B) repeats located in intron 3 and intron 10 of *IKBKG*, respectively (Fig. 1A).

Two benign variants of IP locus, which do not affect the basic structure of the *IKBKG*, were found in healthy parents of IP children with de novo *IKBKGdel* [Fusco et al., 2009, 2010]. These variants are: *MER67Bdup* allele, which contains the duplication of region between MER67B in the *IKBKG* [Variation_74087, Table 3; Aradhya et al., 2001a, Fusco et al., 2009; Conrad et al., 2010], and *IKBKGPdel*, which contains the deletion of the same segment between MER67B in the *IKBKGP*. Both variants are present in the control population with frequency of 1.9% and 2%, respectively (Supp. Fig. S2, Table 3).

Genetic evidence demonstrated that the *IKBKGPdel* and *MER67Bdup* variants should be considered as risk alleles for

Table 3. Large Variants in IP Locus

Genomic variation	Genomic region[a]	Extention (kb)	CNV	Reference
Variation_74087	153,785,404–153,798,129	12.7	dup/del	[Conrad et al., 2010]
IKBKGdel	c.399-?_12601?del	11.7	del	[Fusco et al., 2008]
	153,786,231–153,797,928			[Smahi et al., 2000]
MER67Bdup	c.399-?_12601?dup	11.7	dup	[Aradhya et al., 2001a]
	153,786,231–153,797,928			[Fusco et al., 2009]
IKBKGPdel	153,875,307–153,863,607	11.7	del	[Bardaro et al., 2003]
				[Fusco et al., 2009]
IP-48	153,763,394–153,878,592	115	del	[Fusco et al., 2012]
IP-51	153,763,394–153,828,643	65.2	del	[Fusco et al., 2012]
IP-11	153,757,815–153,821,090	63.2	del	[Fusco et al., 2012]
IP-47	153,757,815–153,803,560	45.7	del	[Fusco et al., 2012]
IP-50	153,769,487–153,796,055	26.6	del	[Fusco et al., 2012]
IP-14	153,774,414–153,796,632	22.2	del	[Fusco et al., 2012]

Dup, duplicated region; del, deleted region; CNV, copy number variation.
[a]Genomic region involved in the variations mapped in UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19).

sporadic IP cases because they promote or facilitate the onset of the pathologic *IKBKGdel* [Fusco et al., 2009, 2010].

Nonrecurrent rearrangements in IP locus

Seven de novo deletions of different lengths (from 4.8 kb to more than 111 kb, Table 3) were reported as nonrecurrent rearrangements in IP locus. They were isolated in IP cases, and their main feature is that they affected not only *IKBKG*, but also the overlapping *G6PD* and the neighboring genes (Fig. 1B) [Fusco et al., 2012a].

The involvement of *G6PD* gene in the deletions found in IP cases raised some important questions about the clinical presentation of patients. Indeed, *G6PD* encodes for the initial enzyme of the pentose phosphate pathway [Luzzatto and Mehta, 1989] and its deficiency (MIM #305900) is the most common X-linked disease reported in human metabolic enzyme defects. The patients, most frequently male, develop a nonimmune haemolytic anemia in response to physiologic stress or to ingestion of triggering substances including some antibiotics and *Vicia faba*, commonly known as faba beans or broad beans. Heterozygous females for *G6PD* deficiency have almost normal G6PD levels due to a selective advantage of cells expressing the wild-type allele [Martini and Ursini, 1996]. Moreover, although a remarkable polymorphism in human populations (>400 variants) and more than 140 different hypomorphic mutations have been reported so far in the *G6PD* gene, large deletions were undiscovered, leading geneticists to suppose that the lack of G6PD was lethal for males [Vulliamy et al., 1993; Luzzatto, 2006; Cappellini and Fiorelli, 2008]. Hence, the survival of IP alleles with *G6PD* deletions in female patients without clinical manifestations matching the classic G6PD deficiency, makes stronger the previous hypothesis suggesting that a very early somatic selection against cells expressing the mutated allele (carrying both *G6PD* and *IKBKG* deletion) has occurred [Fusco et al., 2012a].

Genetic mechanisms producing de novo rearrangements in IP locus

The genomic context analysis of the IP locus reveals that many substrates for different mutational forces such as NAHR, NHEJ, MMEJ, and/or FoSTeS are located there [Fusco et al., 2012a].

In addition, the presence of the LCRs (LCR1 and LCR2) and of the *IKBKGP/NEMOP* pseudogene make risk alleles each benign polymorphism of pseudogene, which can be easily transferred or copied into the *IKBKG*, becoming de novo pathologic mutation. Indeed, the number of de novo mutations in IP is enriched by other alterations that, as reported, derive from the pseudogene through mechanisms such as gene conversion and inversion [Fusco et al., 2009, 2012a].

The NAHR causes the recurrent rearrangements. The NAHR is the most frequent mechanism occurring in the IP locus able to generate aberrant alleles, because it causes the recurrent pathologic deletion (78% of IP cases, *IKBKGdel* allele) and the two benign variants (*MER67Bdup* and *IKBKGPdel*) that are very frequent in control population (1.9% and 2.0%, respectively). These deletions/duplication alleles clustered around the MER67B elements, located as in the *IKBKG* as in *IKBKGP* (>99.1% of identity for 612 bp), suggesting that they are the major substrate for intrachromosomal or interchromosomal NAHR during meiosis germline (Supp. Fig. S3A).

The NHEJ causes the nonrecurrent rearrangements. The NHEJ is, instead, considered the most likely mechanism involved in the onset of nonrecurrent rearrangements. The breakpoint assessment of the junction sequences, essential prerequisite to establish the nature of recombination occurring, showed that the hot-spots of the different nonrecurrent deletions identified in the IP locus were located in a high content of SINEs (33% vs. 15% in IP locus, Supp. Fig. S1C), tandem repeat elements and in the 5′ and 3′ breakpoint junctions mapped an high-density region of repeat sequences and micro-/macro-homologies (16–118 bp; Fig. 1C), well-known substrates for NHEJ [Lieber, 2010]. The heterogeneity and the wide range of the deletions as well as the high content of repeat sequences, micro-/macro-homology regions strongly suggests that DNA repair events occurred, generating different deletions by NHEJ mechanism. The NAHR mechanism cannot be excluded. Indeed, an instance of *Alu–Alu*-mediated recombination was proposed to explain the onset of a nonrecurrent rearrangement (IP-43; Fig. 1B) [Fusco et al., 2012a].

The FoSTeS, MMEJ, and microhomology-mediated break-induced replication cause the new insT/del36 bp mutation. In the present report, we provide evidence on a de novo *IKBKG* exon 4 indel mutation, previously unreported. The sequence analysis at breakpoints/junction enabled us to reveal that a complex rearrangement was present: a deletion of 36 bp and the insertion of a thymine (T) at the junction point (chrX:153786783–153786818, deleted region; UCSC Genome Browser on Human Feb. 2009 GRCh37/hg19) (Fig. 2A). The mutated sequence predicted an altered open reading frame of NEMO/IKKgamma protein synthesis by a premature

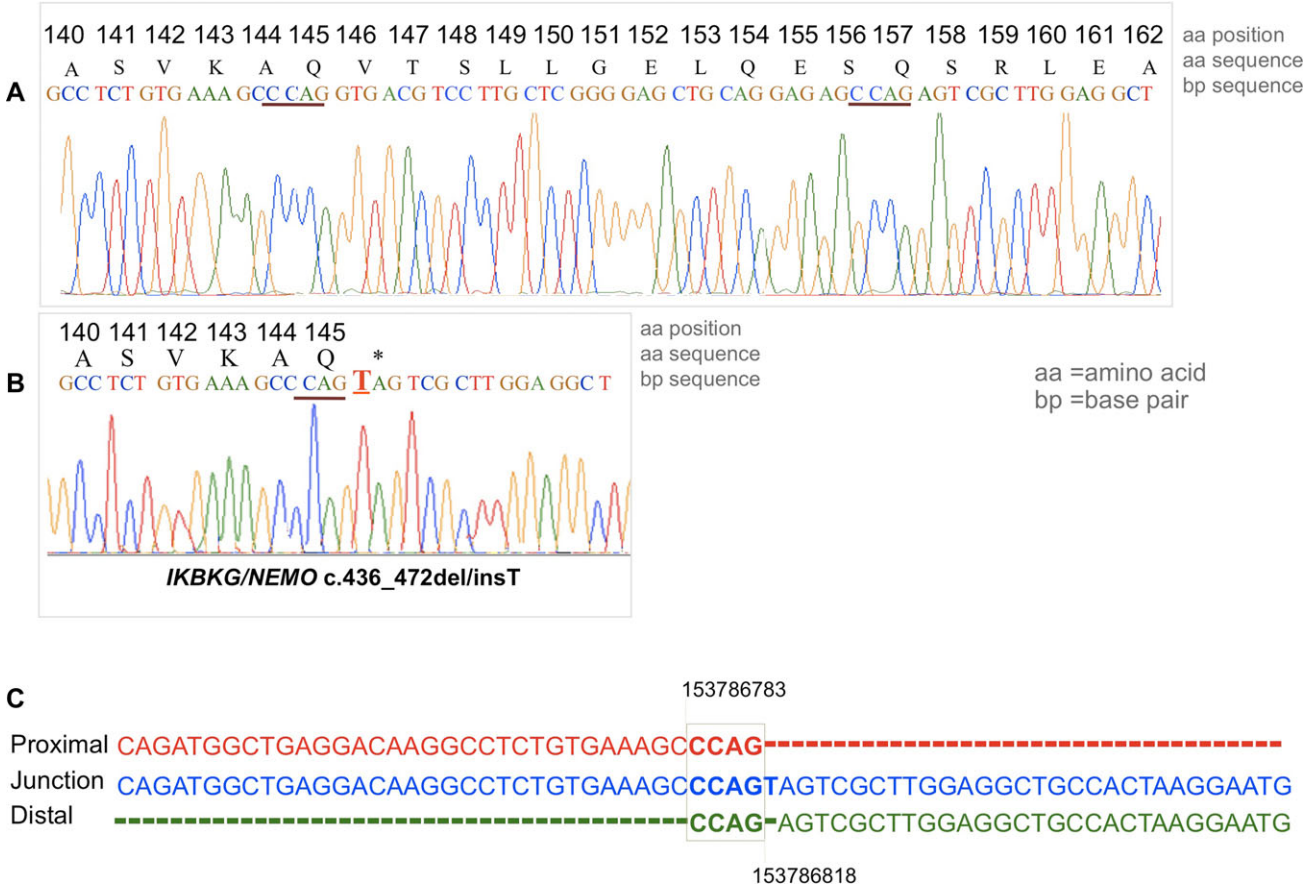

Figure 2. New genomic rearrangement in *IKBKG* gene: c.436_471delinsT. The electropherograms and protein sequences related to wild type (**A**) and to mutated alleles (**B**) isolated from IP-545 patient after cloning of fragment obtained by gene-specific PCR [Bardaro et al., 2003] are shown. The amino acid sequence and position (from 140 to 162) of NEMO/IKKgamma protein is reported on the wild-type allele (**A**). The position of premature stop codon (*) at 146 position is predicted on the mutated allele (**B**). **C:** Alignment of the junction sequences of the *IKBKG* c.436_471delinsT. The proximal and the distal reference sequences separated by the junction sequences (in the middle) are reported. The micro-homology sequences (5′-CCAG-3′, 4 bp) and the T nucleotide insertion, underlined in (**A** and **B**), are boxed in (**C**). The genomic position at junction is shown (UCSC Genome Browser on Human Feb. 2009 GRCh37/hg19).

stop codon (p.Val146X, Fig. 2A, Table 1). The bioinformatic analysis at breakpoint site of the indel mutation, here reported, showed two micro-homology sequences (5′-CCAG-3′) (Figs. 2 and 3) and a non-B DNA structure (tetraplex, favored by stretches of oligonucleotides G) predicted in the deleted region (Fig. 3A). Recently, it has been reported that non-B DNA conformations, repetitive elements, and micro-homology sequences might increase the susceptibility for DNA breakage or promote replication fork stalling [Lobachev et al., 2007; Wells, 2007]. Hence, in the etiology of nonrecurrent microdeletions, MMEJ [Truong et al., 2013; Verdin et al., 2013], FoSTeS [Mirkin, 2013], or Microhomology-Mediated Break-Induced Replication (MMBIR) had occurred [Verdin et al., 2013].

According to these models, we suggest that the mechanism underlying the onset of our indel mutation (c.436_471delinsT, p.Val146X) is a FoSTeS /MMEJ or MMBIR event able to bypass the DNA lesion at stalled fork and to restart the DNA replication (Fig. 3B).

It is likely to suppose that this mutation could have arisen in somatic cells, making the patient a mosaic composition of cells with different alleles. We cannot exclude that this is the case, although no trace of somatic mosaicism was revealed (Supp. Fig. S4).

Inversion and gene conversion events cause de novo mutations. Genetic evidences have demonstrated that variants of pseudogene should transfer to the gene by inversion mechanism mediated by NAHR between LCRs (Supp. Fig. S3B) [Chen et al., 2007].

An instance was reported in one IP family: the unaffected mother had the pseudogene mutation *IKBKGPdel* and the affected daughter had a pathologic *IKBKGdel* on her maternal X chromosome [Fusco et al., 2009]. In that case, it was hypothesized that during the mother's germline development, a NAHR event between the LCRs, mediated by an intrachromosomal loop mechanism, repositioned the exon 4_10 deletion (*IKBKGPdel*) from the pseudogene to the gene making dangerous (Supp. Figs. S3b and S5A) [Fusco et al., 2009, 2010].

In 2001, Aradhya et al. (2001a) have reported that the high degree of identity between LCR1 and LCR2 (>99% identity) was the product of frequent gene-conversion events between the *IKBKG* gene and its pseudogene that homogenize the two sequences. It is likely to assume that inter- or intra-chromatid gene conversion can occur also in IP families generating de novo pathologic alterations (Supp. Fig. S3B). Indeed, in two sporadic IP females carrying de novo *IKBKG* mutations, gene-conversion events were documented [Fusco et al., 2012a]. In both cases, the father had a benign polymorphism in the pseudogene (c.1167delC or *IKBKGPdel*) that perfectly matched the mutation identified in the affected daughters. The haplotype analysis of each member of the family demonstrated that gene-conversion

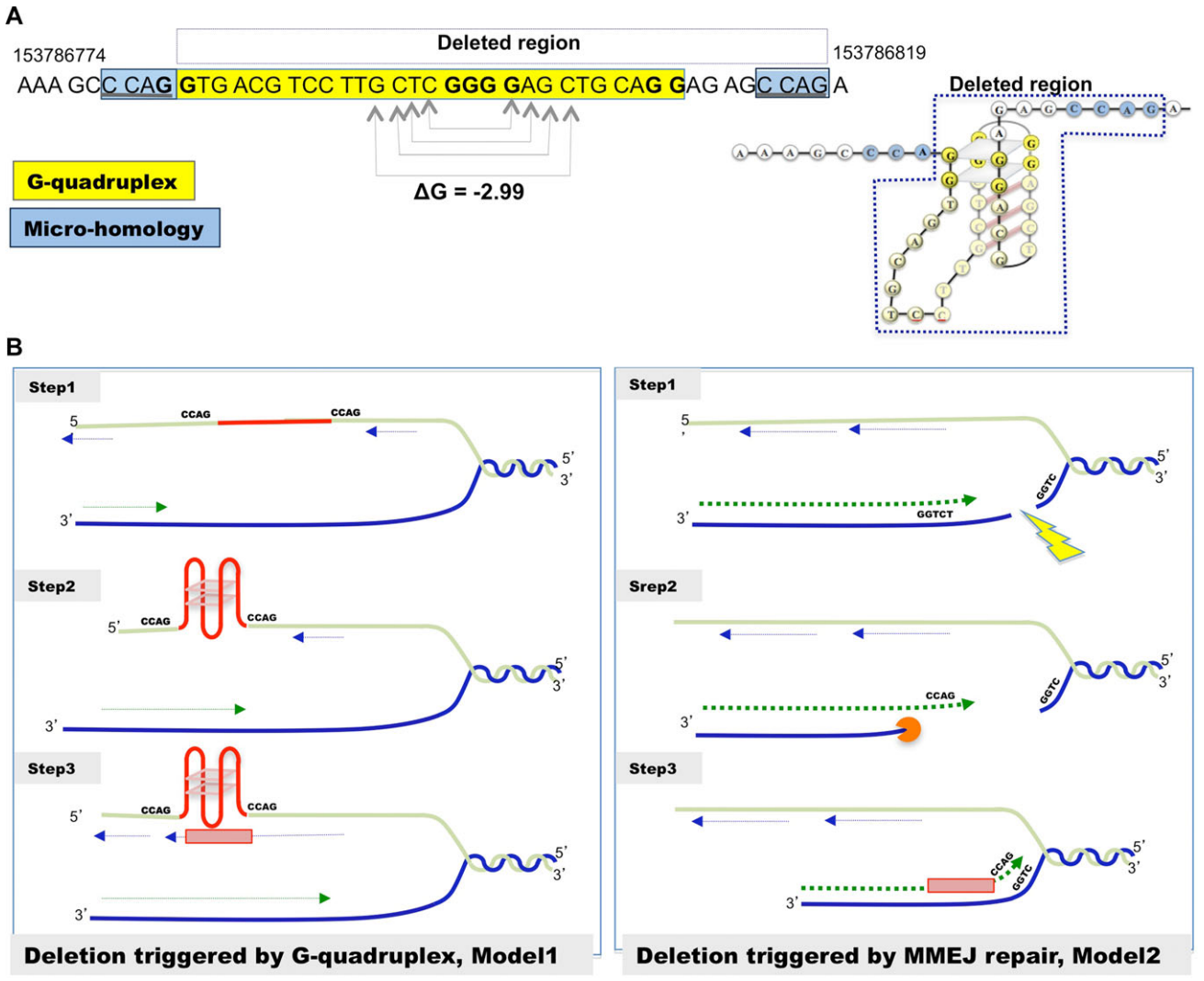

Figure 3. **A:** G-quadruplex predicted structure in the deleted region in mutation c.436_471delinsT. The sequence from chrX:153786774–153786821 (UCSC Genome Browser on Human Feb. 2009 GRCh37/hg19) on Xq28 chromosome region is shown. The deleted region is boxed by dashed lines and in box is indicated the nucleotide involved in G-quadruplex structure predicted by QGRS software (http://bioinformatics.ramapo.edu/QGRS/analyze.php) [Kikin et al., 2006]. The underlined nucleotide indicates the microhomology sequences. The dashed lines indicate the nucleotides that are predicted to give the secondary structure of single-stranded nucleic acids by using *The mfold Web Server* (http://mfold.rna.albany.edu/?q=mfold/dna-folding-form) [Zuker, 2003] and free energy determination is shown (ΔG = –2.99). **B:** DNA-replication models able to explain the onset of *IKBKG*/*NEMO* c.436_472del/insT. During DNA replication (STEP1), the replication fork can stall/collapse and FoSTeS Model1 or MMEJ Model2 can occur. FoSTeS Model1 (on the left): the replication fork can stall, an exposed single-stranded lagging strand template might acquire a secondary structure (G-quadruplex mediated, indicated by DNA hairpin region), which can block the progress of the replication fork (STEP2). The 3′-end then becomes free from its template and after insertion of novel nucleotide may alight on another exposed single-stranded template by sharing the microhomology sequences, restarting the replication (STEP3). MMeJ, Model2: the replication fork can collapse and specific 5′->3′ resection acts (STEP1). The repair of break is mediated by MMEJ mechanism (STEP2). The 3′-end of leading strand may then alight on exposed single-stranded template sequence that shares the microhomology sequence restarting the replication. Then, the pairing of microhomology sequences stabilizes the broken ends and ligation can occur (STEP3).

events in paternal germline occurred, generating de novo altered alleles causing IP (Supp. Fig. S5B).

Biological Relevance

Functional Studies of NEMO/IKKgamma Mutant Proteins

Irrespective of the type of *IKBKG* mutation occurring, X-inactivation skewing, caused by selection against cells expressing the mutation, is often observed in blood cells of IP patients [Sebban and Courtois, 2006]. This aspect of the disease, besides having an impact upon the phenotype developed by IP patients, impairs any further functional analysis of *IKBKG* mutations in cells from female patients. However, many tools are available to perform molecular analysis of the impaired NF-kB function due to IP-associated *IKBKG* mutation.

IKBKG encodes for the NEMO protein (also called IKKgamma), which has a key role in the NF-kB pathway as a regulatory subunit of the IKK complex. Among the variety of stimuli and molecules involved in the activation of the "canonical" NF-kB pathway, the activation of the IKK complex is required for the IkB inhibitor proteins (IkBs) phosphorylation and proteasomal degradation. The

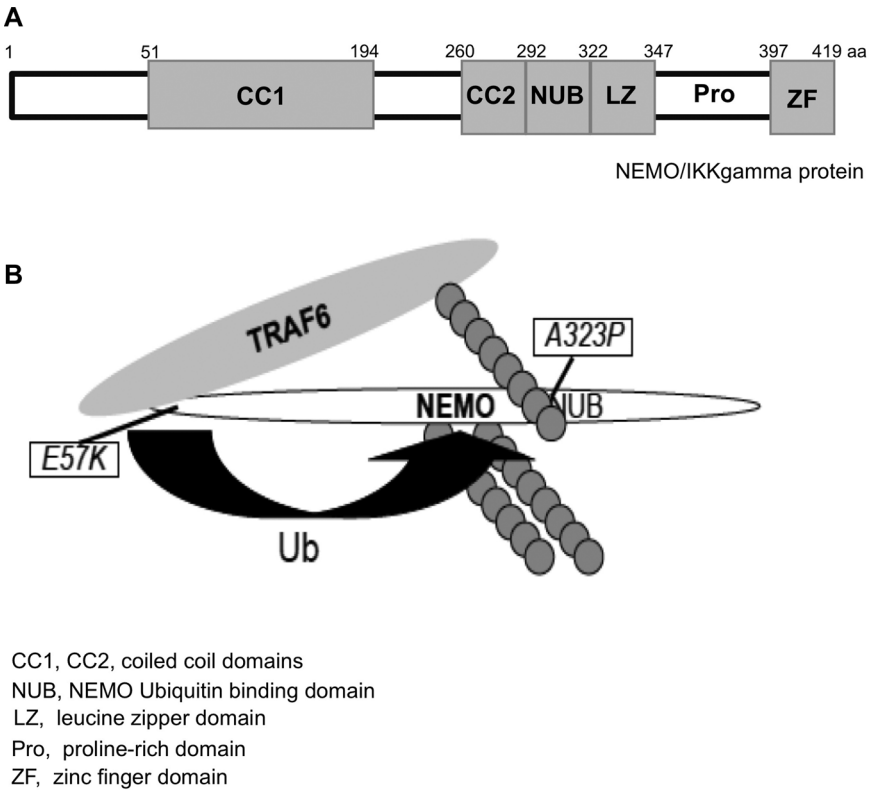

Figure 4. **A:** Schematic representation of NEMO/IKKgamma protein and the protein domains are shown. **B:** Dual mode of recognition between NEMO/IKKgamma and TRAF6. Data gathered from our molecular studies indicate that NEMO/IKKgamma directly interacts with TRAF6 through its N-terminus and recognizes ubiquitinated (Ub) TRAF6 through its NUB domain. IP-associated E57K and A323P mutations distinctly affect these two modes of interaction [Gautheron and Pescatore et al., 2010].

freed NF-kB transcription factors move into the nucleus, where they bind to specific sequences of target genes, encoding essential proteins for innate and adaptive immunity, inflammation and cell survival [Hayden and Ghosh, 2012]. NEMO/IKKgamma does not have enzymatic activity, however, genetic and biochemical approaches have shown that it is absolutely required for IKK activation in response to various stimuli. How NEMO/IKKgamma participates in IKK activation, however, is still unclear. It is known that NEMO/IKKgamma can be modified through posttranslational modifications that regulate its function. They include phosphorylation, sumoylation, or nondegradative ubiquitination [Gautheron and Courtois, 2010]. Adding another level of complexity to the mechanism(s) of IKK complex activation, NEMO/IKKgamma is also an ubiquitin-binding protein. It interacts, through its NEMO Ubiquitin Binding (NUB) domain, with the ubiquitin chain in a variety of signaling cascades, allowing IKK complex recruitment through its affinity for Ub chains [Lo et al., 2009; Grubisha et al., 2010].

IKBKG nonrecurrent rearrangements in IP result in the absence of NEMO/IKKgamma protein, whereas in recurrent rearrangement (*IKBKGdel*), a truncated version of NEMO/IKKgamma (amino acid 1–133) is synthesized, which was revealed by Western blotting of cytoplasmic extracts from primary embryonic fibroblasts of a male aborted fetus carrying this deletion [Smahi et al., 2000]. Interestingly, few missense mutations of *IKBKG* have also been identified as cause of IP, suggesting that distinct molecular abnormalities are sufficient to generate a fully penetrant disease.

Many data on the functional characterization *IKBKG* missense mutations found in EDA-ID male patients were reported, providing crucial information on the NEMO-affected interaction in human disease [Bustamante et al., 2011; Picard et al., 2011]. However, we will here focus our discussion exclusively on the data obtained by the analysis of IP-associated *IKBKG* missense mutations, as the two diseases have nonoverlapping clinical presentation.

Several lines of evidence explained the impaired NF-kB signaling as due to incorrect interaction between IP-NEMO mutant and TRAF6. TRAF6 is a crucial signaling molecule participating in several signaling pathways controlling immunity, osteoclastogenesis, skin development, and brain functions [Wu and Arron, 2003]. TRAF6 works as an E3 ligase responsible for K63-linked NEMO polyubiquitination, which is essential for signaling downstream the IL-1/Toll-like receptor superfamily [Sebban et al., 2006]. In this specific pathway, NEMO is ubiquitinated by TRAF6, in addition to participating in IKK recruitment. Remarkably, two IP mutations of NEMO (p.Glu57Lys and p.Ala323Pro) have been characterized in detail, illustrating the power of using pathology-derived NEMO mutations to gain insights into the IKK/NF-kB activation process (Fig. 4) [Sebban-Benin and Pescatore et al., 2007; Gautheron and Pescatore et al., 2010].

The Alanine 323 to proline (p.Ala323Pro or p.A323P) mutation of NEMO/IKKgamma, which falls in the proximity of the NUB domain, has been identified in a girl suffering from a severe form of IP [Sebban-Benin and Pescatore et al., 2007]. This NEMO/IKKgamma mutation caused a major defect in NF-kB activation, not only downstream of TNFR1 but also in several other signaling pathways (such as TNF-, IL-1-, LPS-, and PMA/ionomycin-induced NF-kB activation) as observed in Nemo(–)cells complemented with the NEMO/IKKgamma-A323P expressing plasmid. The mechanism

underlining this ineffectiveness is the reduced binding of A323P–NEMO to ubiquitinated-TRAF6 and the completely abolished ubiquitination of surrounding lysines. Thus, evidencing that TRAF6 binding and K63-linked polyubiquitination of NEMO/IKKgamma is a key event in IKK activation whose perturbation may cause human pathophysiology [Gautheron and Pescatore et al., 2010]. The combination of genetic and biochemical evidence strongly suggests that ubiquitin-mediated regulation of IKK activation has a critical role in human physiology and that its dysregulation can have devastating consequences [Lo et al., 2009].

Ubiquitin binding and ubiquitination of a protein are often coupled. Similarly, mutations that affect NEMO/IKKgamma ubiquitin binding also affect its ubiquitination, making it difficult to determine whether ubiquitin binding or ubiquitination of NEMO/IKKgamma is important for IKK activation [Skaug et al., 2009].

Such a potentially important interaction between NEMO/IKKgamma and TRAF6 was defined in more detail by uncovering a new binding site at the N-terminus of NEMO/IKKgamma, recognized by the coiled-coil domain of TRAF6. The p.Glu57Lys (E57K) mutation of NEMO/IKKgamma, found in a mild form of IP, resulted in impaired TRAF6 binding and IL-1beta signaling. These data demonstrate that NEMO/IKKgamma/TRAF6 interaction is a crucial step in signaling and its impairment might trigger the disease. Moreover, a fragment encompassing amino acid 57–69 of NEMO/IKKgamma is responsible for binding to the CC domain of TRAF6, which is required for NF-kB activation. The N-ter site of NEMO/IKKgamma appears to work in concert with the previously identified NUB domain, which binds polyubiquitinated chains. This evidence suggested a dual mode of TRAF6 recognition that may allow the design of positive and negative regulators of NF-kB activation, with specificity restricted to TRAF6-dependent stimuli, which uncover many aspect of the IP disease [Sebban-Benin and Pescatore et al., 2007; Gautheron and Pescatore et al., 2010].

Animal Model of IP

Soon after the genetics study in 2000 from the International Incontinentia Pigmenti Consortium showing that the mutation in the *IKBKG* account for the majority of human IP, two reports of mice with targeted *Ikbkg* deletions were published [Makris et al., 2000; Schmidt-Supprian et al., 2000]. As expected, Ikbkg-deficient males died in utero from liver apoptosis at day E12, and heterozygous females were viable. These females displayed skin findings strikingly similar to human IP-lesions on dermatological examination and on histologic study of skin biopsies.

Most importantly, while 55% of the heterozygous females died prematurely with a highly runt appearance, the remaining 45% recovered the cutaneous phenotype that was almost indistinguishable from normal age-matched *Ikbkg*+/+ females. As in human, the surviving heterozygous females were fertile and could transmit the disease to their progeny.

Remarkably, similar skin manifestations have also been observed after invalidating *nemo* or *ikkbeta* specifically in the epidermis, using conditional recombination [Pasparakis et al., 2002; Nenci et al., 2006]. This suggests that keratinocyte dysfunction may be sufficient to trigger the disease. In both strains of mice, it has been shown that IL-1beta and TNF-alpha are likely participants in dermatosis occurrence and development. At very early stages (P2/P3), before detecting any skin abnormalities, an increase of IL-1beta synthesis was observed in the epidermis. Interestingly, such accumulation was not seen with *ikkbeta* KO keratinocytes cultured ex vivo. Later on, at P4/P7, an accumulation of TNF-alfa in the dermis was detected. This cytokine plays a key role in the dermatosis process since crossing the mice with *tnfr-1* KO mice abolishes all its manifestations.

On the cellular side, keratinocytes (detailed above), and macrophages [Stratis et al., 2006] appear to play an important function in IP dermatosis, whereas B and T lymphocytes are not necessary. From these observations, it has been proposed that the mosaic status of the neonatal skin/epidermis of IP females, composed of cells expressing either wild-type or mutant NEMO/IKKgamma, might be a trigger for the whole dermatosis process. Mutant cells, most likely keratinocytes, for reasons that are not yet clear, would start to overexpress pro-inflammatory cytokines such as IL-1 after birth. Then, IL-1beta would induce TNF-alfa synthesis by neighboring wild-type cells, and this cytokine would in turn act on mutant NEMO/IKKgamma-expressing cells, inducing their death and clearance. This sequence of events may explain the final atrophic stage of IP dermatosis linked to lesion disappearance.

TNF- and IL1-mediated inflammation is an essential component of the disease. Therefore, the NEMO/IKKgamma-dependent molecular principles that enable the correct functioning of these NF-kB-independent signaling pathways implicated in the induction of the inflammatory reaction might mediate the triggering of the disease. The identification of player in this signaling will provide putative targets for an early pharmacological treatment of the disease.

Clinical and Diagnostic Relevance

Differential Diagnosis in IP

The diagnosis of IP is fairly easy in the presence of classical features, but can be difficult in cases with partial or nonclassical clinical features, especially in the parents. The demonstration that the disease is caused by mutations in *IKBKG*, has remarkably improved genetic counseling for this disorder and the ability to identify additional signs of the disease, which were previously neglected due to the uncertainty of the IP diagnosis [Fusco et al., 2012b].

The most severe consequences of IP, leading to major morbidity and even mortality, are those affecting the CNS, awareness of which has significantly increased due to the improvement of the method of molecular diagnosis, and has encouraged the epidemiological data collection of IP. Currently, in systematic review among 795 neurologically investigated IP patients, the presence of CNS anomalies is reported in 30.44% of the IP patients, most of them (61.98%) suffered from severe CNS anomalies [Minić et al., 2013a]. The most frequent types of CNS anomalies were seizures, motor impairment, and mental retardation, microcephaly, and rare or unclassified anomalies [Minić et al., 2013a]. In analyzed 139 IP patients, the first neurological manifestations occurred in the first week (58.3%), in the first month of life (66.9%), and in the first year (87.8%) of IP patients [Minić et al., 2013a]. In 89 IP patients, the most frequently registered CNS lesions found using brain imaging methods were brain infarcts or necrosis, brain atrophies, corpus callosum lesions, and rarely diagnosed or unspecified CNS lesions [Minić et al., 2013a]. For the time being, there are several hypothesis explaining pathogenesis of CNS manifestations in IP: apoptosis of neurons and glia cells [Aradhya et al., 2001c], apoptosis of CNS vascular cells [Minic et al., 2013a], disorder of myelinization [Philippe et al., 2013], influences of *IKBKG* gene mutation on VEGF [Aradhya et al., 2001c], and eotaxin expression [Jean-Baptiste et al. 2002].

Currently, no specific treatment is available for IP neurological symptoms and the seizure is treated as in other infantile seizure forms [Scheuerle and Ursini, 2010].

Genotype–Phenotype Correlation in IP

The clinical phenotype of IP is widely variable, even within the same family. It can range from mild skin alterations (mild IP) to stroke and functional CNS abnormalities (severe IP) [Fusco et al., 2004].

Abundant data show that the same *IKBKG* mutation (*IKBKGdel*) might produce mild IP in the mother and severe IP in the daughter in the same family. It has been assumed that such heterogeneity is due to an X-inactivation in heterozygous IP females, coupled with the pleiotropic role of the NEMO. Because the NEMO/IKKgamma protein is involved in a complex signaling pathway that regulates the expression of hundreds of genes, its mutation produces different phenotypic outcomes and may explain the entire spectrum of anomalies seen in IP [Nelson, 2006]. Moreover, as observed in many Mendelian diseases, additional genetic factors, as modifier genes, might in part explain the differences in disease expression in patients carrying the same *IKBKG* mutation (*IKBKGdel*) in different genomic backgrounds.

At the milder end of the disease spectrum, we would expect to find patients who carry missense mutations that only slightly affect NEMO/IKKgamma function. However, the missense mutations are very few in IP thus impairing any statistical analysis of genotype–phenotype correlation. Nevertheless, for those *IKBKG* missense mutations with established NF-kB signaling defect, at least a partial genotype–phenotype correlation can be drawn. The spectrum of clinical outcome may range from very severe phenotype as in the case of p.Ala323Pro mutation, which caused a generalized impairment of NEMO/IKKgamma activity affecting many triggering signaling, to very mild form of IP such as the case of p.Glu57Lys, which presented only an IL-1-specific impairment in vitro. Therefore, we might tentatively conclude that the variability in IP clinical outcome would then result from a combination of the type of mutation, the functional domain affected, X-inactivation, and eventually, the genomic background.

Molecular Diagnostic Strategies in IP

Twelve years of genetic analysis of IP have produced the important assumption that IP belongs to the class of pathologic conditions known as genomic disorders, and that the *IKBKG*_IP locus must be considered a genomic instability region linked to an inherited disease [Fusco et al., 2012a].

As the consequence of this assumption, centers involved in molecular diagnosis of IP have improved their general ability to perform an IP molecular diagnosis, by applying a three-step protocol for the molecular identification of *IKBKG* mutation: (1) recurrent deletion, (2) small exon mutations (indel), and (3) nonrecurrent deletions, at the IP locus. Collectively, this analysis has revealed a *IKBKG* mutation in 88% of IP patients (recurrent deletion in 72%, nonrecurrent deletions in 4%, and small indel/missense in 12%), whereas the remaining 12% of IP patients do not have any identifiable alterations of the *IKBKG* gene. Given that *IKBKG* gene mutations are considered the only genetic cause of IP, we cannot definitively exclude that unexplored regions in the IP locus could have alterations causing IP in the remaining 12% of patients. Moreover, as in about 76% of IP patients, the genetic defect is a copy-number loss of *IKBKG* paralogous copies, a step of quantitative analysis of *IKBKG* copies should be introduced as routine screening in IP molecular diagnosis [Fusco et al., 2012b].

Future Prospects

Here, we have presented a review of past and present findings on the *IKBKG* mutations by exploring with a high accuracy both genomic context in which the mutations occur and the functional effects on the mutated proteins. To these ends, we have compiled a collection of data on published and previously unreported *IKBKG* mutations associated with IP disease.

The availability of a more extensive description of the clinical phenotype of IP patients, together with their mutational genotype as reported in the *IKBKG*-LOVD database, might provide insight into genotype–phenotype correlation. Moreover, genetic studies enable us to understand different mechanisms of mutagenesis that characterize IP to apply this knowledge in genetic diagnosis of disease. On the other hand, further elucidation of the function of selected IP-associated *IKBKG* mutants in several cell lines will provide essential information on the role and on activity of NEMO/IKKgamma that could suggest new targets for the development of therapeutic strategies.

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3.3 Unraveling the Link Between Ectodermal Disorders and Primary Immunodeficiencies

Since both epidermal and thymic epithelium have ectodermal origin, skin abnormalities may represent warning signs of PIDs. This review, published on *International Review of Immunology*, will be focused on PIDs associated to skin and skin annexa abnormalities, highlighting the alarm signs that should lead the clinician to consider a deeper immunological assessment, investigating both molecular and functional aspects. This approach would be very helpful in the early detection and treatment of very complex disorders. In particular, ectodermal dysplasia, hyper IgE syndromes and Nude/SCID syndrome will be discussed.

Conclusive remarks

The association between developmental abnormalities of the skin and immunodeficiencies suggest a role of the skin as a primary lymphoid organ. In support of this hypotesis, there is the evidence that human skin-derived keratinocytes and fibroblasts, are able support the in vitro differentiation into T-lineage committed cells, in the absence of thymic components.

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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-1β, IL-18 and TNF-α) [15]. Impaired IL-1β- and IL-18-dependent induction of IFN-γ, impaired cellular responses to IFN-γ-inducible TNF-α, 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-κB 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-κB pathway are responsible for different forms of HED-ID. Gain-of-function mutations of IκBα are able to enhance the inhibitory capacity of IκBα through the prevention of its phosphorylation and degradation, and result in impaired NF-κB 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-1β and IL-18), and TNFR (TNF-α, LTα1/β2 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 IFNα, 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 *NEM*O 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*, γc, 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

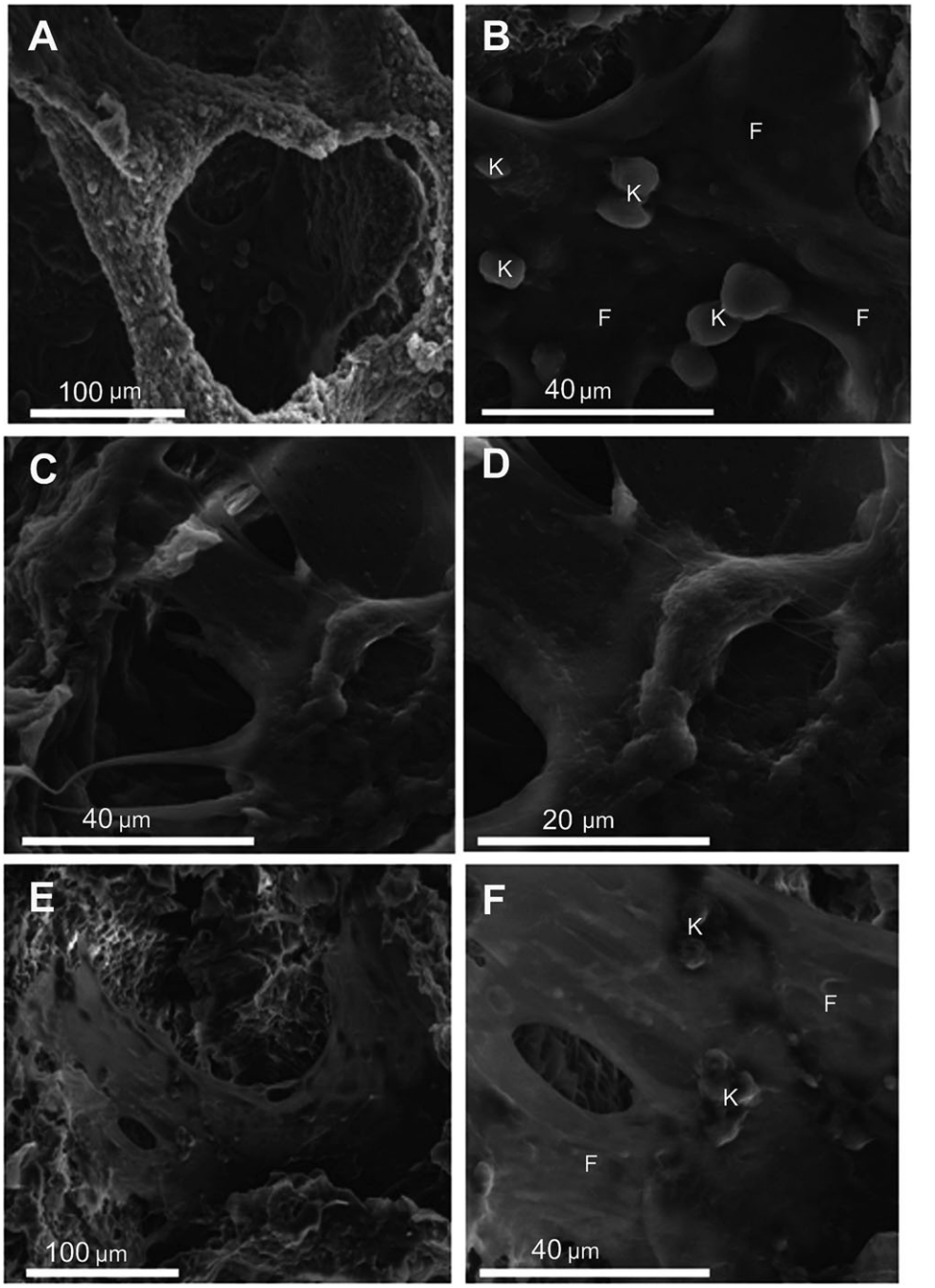

FIGURE 1. Representative scanning electron micrographs of keratinocytes and fibroblasts co-cultured on the PCL scaffold. Expanded fibroblasts and keratinocytes were seeded together onto artificial 3D PCL scaffolds, and after cell infiltration, the structure was studied by scanning electron microscopy (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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3.4 FOXN1 in organ development and human diseases.

The thymus is the primary lymphoid organ that supports T cell differentiation and repertoire selection (163, 164).

Thymus anlagen originate from the third pharyngeal pouch in the embryonic foregut (165, 166). From embryonic day 11.5 in mice and at the eighth week of gestation in humans, the lymphoid progenitor migrate to the thymus (167, 168). Following the entry into the thymus through the corticomedullary junction, the differentiation of T cells require a dynamic relocation of developing lymphocytes within different structures composing this organ lymphoid progenitor cells. The developmental pathway is a multistep process, in which each step is defined by peculiar immunophenotypic patterns: the CD4−CD8− double negative (DN) stage, the CD4+CD8+ double positive (DP) stage, and the CD4−CD8+ or CD4+CD8− single positive (SP) stage. The journey of the immature thymocytes through the thymus plays an important role in the generation of the thymic microenvironment, by promoting the differentiation of stromal precursors into mature thymic epithelial cells TECs (169-172). Appropriate crass-talk between thymocytes and thymic stromal cells is necessary to guarantee the prenatal thymus development, the maintenance of a proper thymic microenvironment, and the efficient T-cell production (173).

FOXN1 is a developmentally regulated transcription factor, which belongs to the forkhead-box gene family, absolutely required for the normal differentiation of hair follicles and TECs (174). During embryogenesis FOXN1 is expressed in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney, and urinary tract. Later

in life, its expression is limited to skin and thymus epithelia. FOXN1 gene spanning about 30 kb (175, 176), has two different noncoding first exons (176), the exons 1a and 1b, that undergo to alternative splicing. The alternative usage of the exon 1a or 1b seems to direct the tissue specificity (177), in that promoter 1a is active in thymus and skin, while promoter 1b is active only in skin. Studies suggest that FOXN1 modulate the thymic stroma differentiation and thymopoiesis through upregulation of the expression of fibroblast growth factor (FGF) receptors (178). Wnt proteins which belong to a family of secreted glycoproteins with important roles in cell-fate specification, upregulate FOXN1 protein expression in both an endocrine and paracrine fashion (179). Even though the role foxn1 in thymus fetal differentiation process has been largely studied, its postnatal role in the mature thymus still remains to be fully elucidated. Foxn1 is expressed in all TECs being required for the initial phase of their differentiation (180-182), and for the induction of both cortical and medullary differentiation (183, 184). The inborn null mutation in foxn1 (185) halts thymic development at a rudimentary stage, in which the epithelial cells are not able to induce the entrance of hematopoietic precursor cells (HPCs) into the epithelial cluster and thus preclude the generation of thymocytes (186), suggesting a failure in thymocytes-epithelial crosstalk (180, 187).

The human equivalent of the “nude” murine phenotype has been described for the first time in two sisters, more than 30 years after the initial mouse description. Subsequently, it was associated to alterations in FOXN1 gene. The human Nude/SCID is an autosomal recessive disorder (188), characterized by a complete absence of the thymus and consequent T-cell immunodeficiency. This immunodeficiency is similar to the classical SCID phenotype,

and more severe than Di George Syndrome. Along with the severe infections, other features of the syndrome are ectodermal abnormalities, as alopecia and nail dystrophy (189). The nail dystrophy has been described also in subjects carrying the heterozygous genetic alterations. FOXN1 is expressed in the nail matrix and is involved in the maturation of nails (189). Anomalies of brain structures, have also been described in Nude/SCID, suggesting a potential role of this transcription factor in brain embryogenesis. The brain anomalies have been considered potentially responsible for the high rate of in utero mortality observed in a geographic area with high frequency of FOXN1 alteration (190). Human FOXN1 deficiency impairs the development of the T-cell compartment since the 16th week of gestation (191). The other hematopoietic compartment including stem cells, B, and NK lymphocytes are not impaired. Both CD4+ and CD8+ cells are profoundly reduced cells even though FOXN1 deficiency affect more CD4+ cell compartment than CD8+. CD4+CD45RA+ naive cells are completely absent (191) while CD8 cells coexpressing CD3 are detectable, even though at very low levels and only few CD3+CD8+CD45RA+ naıve cells can be detected (191). The number of lymphocytes expressing TCRγδ is usually normal (191). TCR gene rearrangement, occurs to some extent, suggesting the existence of an extrathymic and FOXN1-independent site of differentiation. However, the low number of T cells, which escape the blockage, are unable to sustain a productive peripheral immune response.

Because of the significant expression levels of FOXN1 in skin elements, keratinocytes have been successfully used to support a full process of human T-cell development in vitro, resulting in the generation of mature T cells from HPCs. This finding would imply a role for skin as a primary lymphoid organ (192). Our and other groups reported

on the ability of human skin-derived keratinocytes and fibroblasts co-cultured on 3D poly ε-caprolactone scaffold to support in vitro HSC differentiation into T-lineage committed cells. The ex vivo reproduction of microenvironments of the native tissue through approaches based on the use of porous and biodegradable matrices is useful to repair or replace tissues damaged at a molecular or functional level (193). This approach has been successful in achieving the regeneration of many tissues, such as skin (194), cornea (195), blood vessels (196) and in the bone replacement process (197, 198). Optimal scaffold materials should exert the properties of excellent biocompatibility, suitable microstructure, controllable biodegradability and suitable mechanical properties to sustain and facilitate a proper intercellular connection (199, 200). Scaffold surfaces need to be organized to allow optimal cell–cell contact, growth, maintenance of morphology and viability over time to meet the demands of the specific application (201). In congenital immunological disorders and, in particular, in athymic disorders, a scaffold mimicking the 3D structure of primary lymphoid organs may be potentially used for the differentiation of haematopoietic cell precursors and, eventually, allows the re-setting of immunological response through functional or molecular manipulation of precursor cells. In keeping with this, it has been recently documented that a 3D tantalum coated carbon matrix is able to support the development of functional T cells from haematopoietic precursor cells in the context of a heterogeneous multicellular system (202), even though the capability of the system to ensure a complete process, resulting in mature type T cells, is still under debate (203).

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ARTICLE

FOXN1 in Organ Development and Human Diseases

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FOXN1 gene belongs to the forkhead box gene family that comprises a diverse group of "winged-helix" transcription factors that have been implicated in a variety of biochemical and cellular processes, such as development, metabolism, aging and cancer. These transcription factors share the common property of being developmentally regulated and of directing tissue-specific transcription and cell-fate decisions. *Foxn1* is selectively expressed in thymic and skin epithelial cells, where it acts through its molecular targets to regulate the balance between growth and differentiation. In particular, *Foxn1* is required for thymic epithelial patterning and differentiation from the initial epithelial thymic anlage to a functional cortical and medullary thymic epithelial cells (TECs) meshwork necessary for the crosstalk with the lymphoid compartment. A mutation in *FoxN1* generates alymphoid cystic thymic dysgenesis due to defective TECs, causing primary T-cell immunodeficiency, named Nude/SCID syndrome, and leads to a hairless "nude" phenotype in both mice and humans. This immune defect represents the first example of a Severe Combined Immunodeficiencies (SCID) phenotype not primarily related to an abnormality intrinsic of the hematopoietic cell, but rather to a peculiar alteration of the thymic epithelia cell. This review focuses on the key role of FOXN1 in cell development and its clinical implication in humans.

Keywords FOXN1, immunodeficiency, Nude/SCID, skin, thymus

FOXN1 gene belongs to the forkhead gene family that comprises a diverse group of "winged-helix" transcription factors that have been implicated in a variety of biochemical and cellular processes, such as development, metabolism, aging and cancer [1, 2]. It was first well characterized both in mouse and humans by Schropp et al. in 1997. First designated as winged helix nude gene, because of the presence in its sequence of a forkhead domain (*Whn*), it has been then renamed forkhead box n1 (*FOXN1*). The human *FOXN1* gene is localized on chromosome 17q11–12, spanning about 30kb and being closely linked to the neurofibromatosis-1 gene [3]. Similarly to the mouse *Foxn1* gene, it contains eight coding exons and two different first exons, exons 1a and 1b, that undergo to alternative splicing to either of two splice acceptor sites of the exon 2, located upstream of the initiation codon. Exon 1a and exon 1b do not contribute to the protein sequence but, interestingly, they are under the control of two promoters, which confer *FOXN1* gene tissue specificity, in that the promoter 1a is active both in thymus and skin and the promoter 1b only in the skin [3, 4].

FOXN1 gene encodes an evolutionarily high conserved forkhead/winged helix transcription factor [5], which contains 648 amino acids and exerts an 85% homology

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between rodents and humans [3]. Structural characteristics of the protein include a transcriptional activation domain, encoded by exons 8–9 and a forkhead DNA binding domain, encoded by three exons (5–7), this structure being peculiar of the forkhead/winged helix transcription factors [3]. The presence of the transactivation domain in *FOXN1* C-terminal region has been first proven by Brissette et al. [6] and then confirmed by the study of Schuddekopf et al., in which it was shown that the domain consists of approximately 50 amino acids between aa 511 and 563, rich of acidic residues [7]. Using site-directed mutagenesis, the authors replacing aspartic acid residues with alanine, found that these acidic residues are essential for the transactivation activity of the protein. Notably, not only the structural integrity of the amino acidic sequence, but also the physical proximity of both domains are essential for FOXN1 transcriptional activity [8].

Postnatally, *Foxn1* gene is mainly expressed in thymic epithelial cells (TECs), some keratinocyte populations and hair follicles, where it acts through its molecular targets to regulate the balance between growth and differentiation [6, 9, 10]. It exerts its function after activation through phosphorylation that promotes its nuclear translocation [11–13]. Into the nuclei, it interacts with DNA as a monomer through its forkhead box, but the target genes and the specific biochemical mechanism of interaction with the promoter regions remain to be elucidated [3, 14].

The molecular mechanisms by which FOXN1 expression and activity are regulated are not fully understood, even though evidence suggests that FOXN1 expression is strongly regulated by wingless (Wnt) proteins [15], a family of secreted glycoproteins that play a role in the cell-fate specification [16], and bone morphogenetic protein (BMP) signaling, required for a normal thymus development [17], in both autocrine and paracrine fashion (Figure 1) [15]. Foxn1 target genes have not been completely identified due to technical difficulties in isolating physiologically intact TECs at different stages of the differentiation process, even though growing evidence is now available documenting that several genes are molecular target of Foxn1 (Figure 1), including Notch ligands, *DLL-1* and *DLL-4* [18] and the chemokines, *CCL25* and *CXCL12* [19].

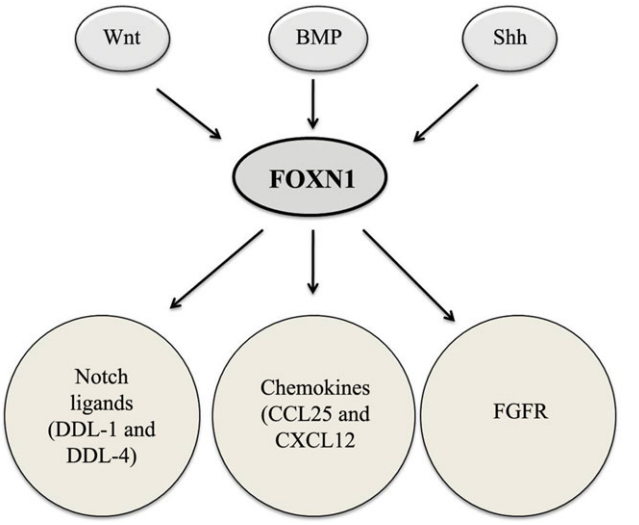

FIGURE 1. FOXN1 regulators and target genes. FOXN1 expression is regulated by wingless (Wnt) proteins, sonic hedgehog (Shh) and bone morphogenetic protein (BMP) signalings. Several genes are molecular target of Foxn1, including Notch ligands, Delta like ligand- (DLL-)1 and DLL-4, the chemokines CCL25 and CXCL12 and the fibroblast growth factor receptor (FGFR).

Moreover, it has been documented that FOXN1 up-regulates the expression level of fibroblast growth factor receptor (FGFR), which are able to regulate the differentiation of thymic epithelia and thymopoiesis, as well [16].

Even though the complete pattern of FOXN1 expression and its role are not yet completely unraveled, the knowledge of the prominent role of FOXN1 came out from studies on nude mouse and the human equivalent model determined by gene alterations. Mutation in *Foxn1* generates alymphoid cystic thymic dysgenesis due to defective TECs [5, 9] and leads to a hairless phenotype in both mouse and humans [10].

THE ROLE OF FOXN1 IN THE THYMUS

The thymus provides a specialized microenvironment for the development and selection of mature T cells bearing the T-cell receptor (TCR) complex [19, 20]. The thymic microenvironment is composed primarily of an integrated meshwork of TECs organized in three-dimensional (3D) architecture. TECs are differentiated in cortical (cTECs) and medullary epithelial cells (mTECs), required for promoting most stages of the thymocyte differentiation [21]. The intrathymic development of T cells consists of discrete phases that require a dynamic relocation of developing lymphocytes within multiple architectural structures of this organ. In the T-cell developmental pathway, hematopoietic progenitor cells (HPCs) emigrate from the bone marrow to the 3D thymus, where the developing T-cell precursors, namely, thymocytes, interact with TECs and other thymic stromal cells (TSCs). Thymocytes relocate within the organ, proliferate and acquire lineage specification. At molecular level, TCR rearrangement occurs resulting in the emigration from the thymus of a fully differentiated mature T cell [22, 23].

In particular, the epithelial cell-autonomous gene *Foxn1* is required for thymic epithelial patterning and differentiation from the initial epithelial thymic anlage to a functional cTEC and mTEC meshwork during crosstalk with the lymphoid compartment. In particular, the development and maturation of TEC subsets during the thymus organogenesis occurs through two genetic stages [9, 24], first stage being *Foxn1*-independent and under the control of genes such as *Hoxa3* [25] and *Tbx1* [26], during which induction and outgrowth of the thymic epithelial anlage from the third pharyngeal pouch take place. In *Foxn1*-dependent step, precursor epithelial cells differentiate into mature and functional cTECs and mTECs from the same bipotential TEC progenitor [24, 27–29]. Of note, Foxn1 is expressed in all TECs during embryogenesis, but not in all TECs of the adult thymus [30], indicating that the gene is strictly developmentally regulated. However, studies on K5- and K18-CreERT-mediated *Foxn1*-deleted mouse models have revealed that *Foxn1* plays a different role in mTEC and cTECs. In particular, during the postnatal life, mTECs, expressing keratins type K5 and K14, which are similar to epithelial stem cell markers and exhibit progenitor activity in the skin and mammary gland [31, 32], are affected in the case of loss of *Foxn1*. Conversely, cTECs, whose markers are the keratins K8 and K18 expressed in terminally differentiated epithelial cells in the apical layer of stratified squamous skin epithelium, are not sensitive to the loss of *Foxn1* [33]. Thus, this evidence suggests that cTECs and mTECs are not equally *Foxn1*-dependent and cTECs are insensitive to *Foxn1* missing. Since Foxn1 is widely expressed during embryogenesis, its expression in the adult thymus reveals the presence of TEC progenitors [34], expressing $K5^+/K14^+$ markers, which support TEC homeostasis in the adult thymus.

Of note, a new component of proteasome, the β5-thymus (β5t) subunit, selectively expressed in the cTECs has been identified. β5t subunit leads to the production of peptides presented by class I MHC molecules, thus playing a pivotal role in generating the immunocompetent $CD4^-CD8^+$ T cells. In murine cTECs, the expression of β5t is

strictly dependent on *Foxn1*, which might regulate β5t transcription. These data are in keeping with the central role of this transcription factor in the stroma functionality [35].

Additionally to the well-defined role of the Foxn1 to regulate the thymic epithelial patterning and differentiation, it has recently been demonstrated that Foxn1 is also involved in the 3D thymic microstructure morphogenesis and maintenance [36], which is very important for the functionality of the thymus.

Eventually, Foxn1 must be considered as a prime downstream mediator of agents or pathways also capable to induce thymic involution or rebound. Normal aging is accompanied by thymic involution, reduced thymocyte output and reduced expression of Foxn1 [37, 38]. It has been shown that the progressive loss of Foxn1 causes accelerated premature thymic involution, with gross and microanatomic changes similar to age-related involution [38]. Furthermore, intrathymic injection of *Foxn1* cDNA to aged mice increased thymic size and thymocyte numbers [38]. Consistent with these results, down-regulation of Foxn1 occurs early in thymic involution [37], while transgenic overexpression of Foxn1 delays this process [39].

Of note, most of the pathogenic mechanisms underlying a primary T-cell disorder are related to molecular alterations of genes selectively expressed in hematopoietic cells. However, since the differentiation process relies on a proper crosstalk among thymocytes and thymic microenvironment, a severe T-cell defect may also be due to an alteration of the stromal component of the thymus [40].

THE ROLE OF *FOXN1* IN THE SKIN

FOXN1 gene expression in the skin, as also seen in the thymus [9], is restricted to epithelial derived cells [41]. Using *in situ* hybridization and a murine model in which a beta-galactosidase reporter gene was placed under the control of the wild-type *Foxn1* promoter, *Foxn1* gene was found to be expressed since Day 13 of gestation in the nasal region of mice. Then, *Foxn1* expression becomes detectable in developing whisker pads, nail primordial, hair follicles of eyebrows and the epidermis of mouth, nose, ears and tail. At last, *Foxn1* expression involves the entire skin, particularly interfollicular epidermis and hair follicle [41]. A detailed analysis of the *Foxn1* expression in mouse skin has revealed a specific pattern of expression, suggesting its involvement in the regulation of cell growth and differentiation.

In the interfollicular epidermis, which is composed by four cell layers (basal, spinous, granular and cornified), Foxn1 is mainly expressed within the nucleus of the cells in the first suprabasal layer. Its expression in these cells corresponds to the onset of terminal differentiation, having the cells left the cell cycle and started the full program of differentiation [41]. However, Foxn1 expression has been found, along with Ki-67 expression, also in rare cells in the basal layer. These findings allow to speculate that the double positive cells ($Foxn1^+$ $Ki\text{-}67^+$) could be markers of the very first stage of terminal differentiation, being daughter cells of transient amplifying keratinocytes, which are committed to differentiate and are just leaving the cell cycle and the basal layer [42].

In the hair follicle, Foxn1 is expressed in the supramatrical region. In particular, during hair follicle morphogenesis, its most prominent expression has been detected in the precursor cells of the inner root sheath and hair shaft, in the outer root sheath and in the periphery of the matrix [41, 43]. All these cells derive from the matrix cells after hair bulb formation and are characterized by the end of proliferation, in order to achieve their terminal differentiation [44]. After hair follicle formation, a cycle consisting of three phases takes place: anagen, hair follicle growth; catagen, hair follicle regression; telogen, resting period. Notably, Foxn1 expression is strongly dependent

on the phase of hair cycle. Indeed, the gene transcript was easily found only during anagen, while it was poorly or not at all detectable in the two further phases [43].

As far as concerned FOXN1 function in the skin, its role, its target genes and the molecular mechanism of action still remain to be fully clarified. The observation that Foxn1 is mainly expressed in the keratinocytes of the first suprabasal layer, together with the findings that nu/nu mouse keratinocytes showed a much higher susceptibility compared to wild-type keratinocytes to the growth-arresting effect of phorbol ester 12-*O*-tetradecanoyl-13-acetate (TPA), allows to speculate that FOXN1 is a key transcription factor involved in the regulation of keratinocytes growth and differentiation [6]. In addition, an increased proliferation and an impaired keratinocyte differentiation have been found in interfollicular epidermis, hair follicle and urothelium of mice, which overexpress Foxn1 in terminally differentiating cells [45]. Strong evidence suggests that Foxn1 is involved in keratinocyte differentiation through regulation of target genes, such as protein kinase C (*PKC*) and protein kinase B (*AKT*). Indeed, mouse keratinocytes, missing functional Foxn1, showed an up-regulation of PKC activity, while the overexpression of Foxn1 led to the suppression of PKC activation and inhibition of keratinocyte differentiation [46]. In addition, evidence indicates that PKC is a potent inhibitor of human hair follicle growth in vitro [47–49]. Janes et al. have investigated the function of FOXN1 in the regulation of interfollicular epidermis by means of cultures of primary human epidermal keratinocytes. They found that FOXN1 was responsible of initiation of keratinocyte differentiation but it was not sufficient to induce the final stages of terminal differentiation. Indeed, in the reconstituted human epidermis only differentiating cells of the spinal layer could be detected, while cells of the granular and cornified layers are lacking. Moreover, using microarrays analysis, approximately 30 genes have been identified, which are up-regulated following FOXN1 activation during keratinocyte differentiation. Among these genes, the serine/threonine kinase Akt is able to trigger the later stages of differentiation, leading to the development of the granular and cornified layers. Together all these findings allow to hypothesize that while FOXN1 promotes the early stage of keratinocyte differentiation, the increasing levels of AKT and its subsequent activation may bring to completion the process of terminal differentiation [50]. In keeping with this, keratinocytes from nude mice express lower level of keratin 1 (a marker of the early stages) [6], whereas overexpression of Foxn1 increases the expression keratin 1 [45, 51]. The acidic hair keratin 3 (mHa3), the murine ortholog of the human acidic hair keratin 3 and Foxn1 are coexpressed in the same anatomic structure. Reduced levels of mHa3 and other hair keratins were found in nude mice epidermis [4, 43] and transfection of FOXN1 in HeLa cells (tumoral cells derived from human cervical carcinoma) induces a high expression of mHa3, mHb3 and mHb5, hair keratins normally not expressed in this cell line [8].

Furthermore, it has been demonstrated, by using an engineered-keratin-5-driven *Foxn1* (K5-*Foxn1*) transgenic (Tg) mouse, that Foxn1 provides the necessary cues to transfer the pigment from melanocytes to keratinocytes of the hair shaft cortex, this effect being mediated by Fgf2 [52]. The *Foxn1*-null nude mouse completely lacks pigmentation in the hair cortex, while the K5-*Foxn1* Tg confers ectopic acquisition of the pigmentation in hair cortical cells.

CLINICAL IMPLICATIONS OF *FOXN1* MUTATION IN HUMANS: THE NUDE/SCID MODEL

The absence of FOXN1 transcription factor results, both in mice and humans, in congenital athymia and hairlessness. In man, the phenotype has been described in association with a C792T homozygous transition in the FOXN1 gene, which resulted in the nonsense mutation R255X in the exon 4 (formerly exon 5), with a complete absence of

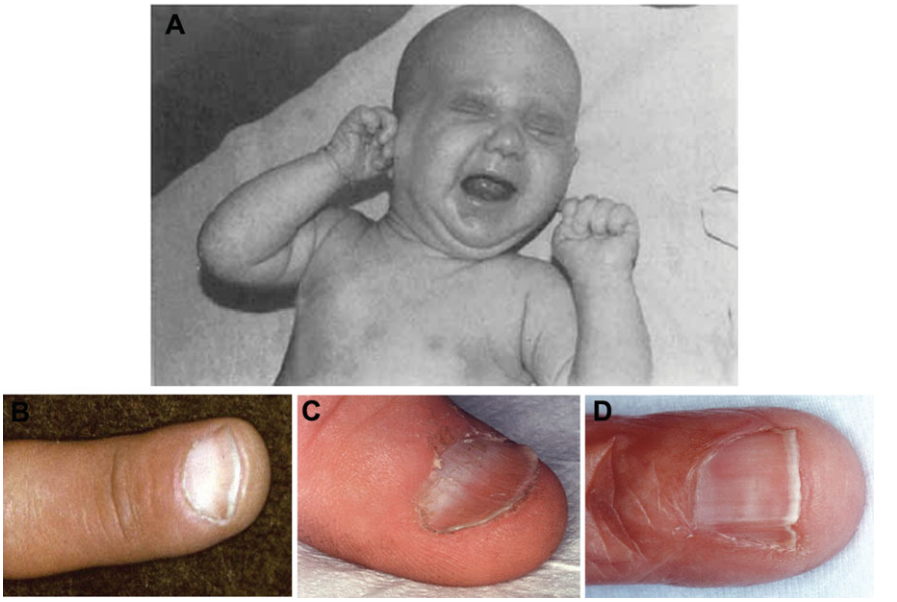

FIGURE 2. Hallmarks of the human Nude/SCID phenotype. (A) Alopecia of the scalp, eyebrows and eyelashes. Nail dystrophy patterns: (B) leukonychia, (C) koilonychias and (D) canaliform dystrophy.

a functional protein [53], or with a C987T (R320W) transition in the exon 6, resulting in a missense mutation of the DNA binding domain [54]. In particular, the genetic alteration of the transcription factor, inherited as an autosomal recessive disorder, leads to a severe T-cell immunodeficiency, congenital alopecia of scalp, eyebrows and eyelashes (Figure 2A). This phenotype, referred to as Nude/SCID (MIM 601705; Pignata Guarino Syndrome), was described for the first time in humans in 1996 in two sisters originating from a small community in the south Italy [55]. This immune defect represents the first example of a Severe Combined Immunodeficiencies (SCID) phenotype not primarily related to an abnormality intrinsic of the hematopoietic cell, but rather to a peculiar alteration of the TEC [40, 55–57].

This phenotype is widely accepted as the human equivalent of the similar murine phenotype, reported for the first time by Flanagan in 1966 [58]. Over the time, the DiGeorge syndrome (DGS), similarly characterized by thymic hypo/aplasia with subsequent impaired thymocyte development, was erroneously considered the human counterpart of nude mouse phenotype. The molecular defect responsible for the DGS is a 22q11.2 region deletion in which *Tbx1*, the gene putatively responsible for the athymia, is located. A DGS-like phenotype could also be due to other molecular defects, such as the deletion of a critical region on chromosome 10p13/14 [59, 60]. Moreover, the CHARGE syndrome, due to mutations in the *CHD7* gene encoding for a chromodomain helicase DNA-binding protein, shares considerable phenotypic overlap with DGS [61]. The spectrum of DGS defects is very heterogeneous and, in most cases, the phenotype is mild [62]. In the majority of patients with DGS, referred to as "partial," the T-cell pool is usually normal or, in very few cases, low normal, and T cells are only rarely poorly responsive to common mitogens, while patients affected with the "complete" form of the disease (accounting for 0.5%–1.5% of patients) have a severe T-cell immunodeficiency, with a naïve T-cell pool and mitogen responsiveness usually absent [63–65]. Thus, even though the Nude/SCID and a few DGS share the absence of the thymus, by a clinical and immunological point of view there are several differences, which lead to consider these disorders two completely distinct entities.

By contrast, Nude/SCID patients exhibit a quantitative and qualitative T-cell defect much more severe than that observed in DGS. The Nude/SCID immune deficiency is characterized by a severe blockage of the T-cell differentiation and absence of proliferative response to the common mitogens [55]. Naïve T cells are lacking in the peripheral blood, as a consequence of the absence of a T-cell differentiation process. In DGS, differently from Nude/SCID patients, $CD4^+CD45RA^+$ naïve cells are usually found, suggesting that a certain level of thymic functionality is still present also in the complete forms of the syndrome. The comparison of the clinical phenotype of the two syndromes reveals that in DGS hairlessness is missing and gross abnormalities of skin annexa are lacking, differently from Nude/SCID. As above described, the transcription factor FOXN1 is also expressed in the epithelial cells of the skin, where it participates to the homeostasis between growth and differentiation. This evidence argues in favor of the essential role of FOXN1 in T-cell development and leads to suggest that skin might express a major role as an alternative primary lymphoid organ. In keeping with this, it has been recently documented that in the absence of thymic cellular epithelial elements, skin-derived keratinocytes and fibroblasts seeded on the 3D scaffold, by mimicking the configuration of the thymus, are able to support the differentiation of stem cells into T-cell precursors [66].

By taking advantage of the congenital athymia, the human Nude/SCID phenotype was used as disease model to investigate the development of the T-cell compartment in the absence of a functional thymus. The results obtained from these studies revealed the presence of some circulating T cells of non-maternal origin in patients carrying alterations of FOXN1, thus raising important questions regarding the site of differentiation of these cells. These cells have been shown to be predominantly double-negative (CD4negCD8neg, DN) $\alpha\beta$ T cells and to exhibit a regulatory-like T-cell phenotype ($FoxP3^+$). One hypothesis was that a thymic rudiment could persist, allowing a partial T-cell development, but with alterations in positive/negative selection, as suggested by the expansion of DN $\alpha\beta$ and $FoxP3^+$ T cells [67]. Further evidence indicates that the *TCRBV* spectra-type repertoire in Nude/SCID patients is oligoclonal in both prenatal and postnatal life [54, 68]. Further studies aimed at characterizing the phenotype of lymphocytes in the Nude/SCID model in human prenatal life revealed that *FOXN1* alteration leads to a total blockage of $CD4^+$ T-cell maturation and severe, but not total, impairment of $CD8^+$ cell differentiation, with an apparent trend toward a $\gamma\delta$ T-cell production. This finding thus suggested a crucial role of this gene in the early prenatal stages of the T-cell ontogeny in humans [68]. Of note, different mutations in the same gene lead to different immunological phenotypes. No circulating T cells were found in a patient with the C987T (R320W) substitution identified in the exon 6 of the human FOXN1 gene, resulting in a missense mutation of the DNA binding domain [54]. These partially divergent features raise intriguing arguments about the role of FOXN1 in the T-cell development, which deserve further exploration using mouse models and comparative structural studies [4, 8, 9].

To date, there is no curative therapeutic approach for Nude/SCID syndrome. Bone marrow transplantation has been performed in one child with the Nude/SCID phenotype due to FOXN1 deficiency but without the production of the naïve T-cell pool and long-lasting immunological reconstitution [69]. This would be expected given the absence of a functional thymus and to the necessary role of the FOXN1 transcription factor for a proper T-cell development. Thus, because of the athymia in FOXN1 deficiency, an attempt to use thymus transplantation was chosen to obtain an immune reconstitution. Remarkably, naïve T cells with a diverse TCR repertoire were generated, which paralleled the normalization of T-cell proliferative responses and Ig levels. This therapeutic intervention was also useful to achieve the clearance of the ongoing disseminated infections [54]. Of note, in one patient undergone to thymus transplantation, it

has been described, for the first time, an unusual leukoderma, which the authors hypothesized as related to FOXN1 deficiency [70]. Upon transplantation of FOXN1 competent thymic epithelia, also the peripheral pool of FoxP3$^+$ T cells normalized, while the number of circulating DN$\alpha\beta$ T cells remained constantly high up to 6 years after transplantation [67]. This finding raises the question about the origin of DN$\alpha\beta$ T cells in that their reduced sjTREC levels, used as a marker of thymic functionality, suggest that they were not produced after transplantation [67], and that they probably did not require a functional thymus to develop. The evidence that other sites, alternative to the thymus, could sustain T-cell development and differentiation also derives from the identification, in a Nude/SCID fetus, of a limited number of CD8$^+$ cells, not expressing the CD3 and bearing TCR$\gamma\delta$, thus suggesting an extrathymic origin for these cells [68].

Nude/SCID phenotype is also characterized by nail abnormalities. Nail dystrophy in mice and humans is a feature of the syndrome. In man, also heterozygous gene alterations are associated with minor ectodermal anomalies, as well. Leukonychia or koilonychia ("spoon nail") are the more frequent abnormalities, the former being characterized by the typical arciform pattern resembling a half-moon and involving the proximal part of the nail plate (Figure 2B), the latter being characterized by a concave surface and significant thinning of the nail plate (Figure 2C) [71]. Also canaliform dystrophy and a transverse groove of the nail plate (Beau line) are observed, but less frequently (Figure 2D) [71]. These features are in keeping with the observation that FOXN1 is expressed in the nail matrix, thus being involved in the nail maturational process [41, 72]. Therefore, nail dystrophy could be considered an important sign to recognize heterozygous subjects [71, 73].

It should be noted that recently, multiple-site neural tube defects including anencephaly and spina bifida have been reported in a human fetus homozygous for the mutation R255X [74]. However, the analysis of a second Nude/SCID fetus did not reveal any gross abnormality in the central nervous system (CNS) anatomy but only the absence of the corpus callosum and cavum septi pellucidi (CSP), and an enlargement of the interhemispheric fissure. Of note, FOXN1 is expressed in murine epithelial cells of the developing choroids plexus in the embryonic brain [74], thus suggesting a possible role of FOXN1 in the development of the CNS in a similar fashion to other FOX family members, such as FoxP1, that helps Hox proteins regulate the genes, controlling the motor-neuron diversification [75, 76]. However, these variable abnormalities reported in the Nude/SCID phenotype suggest that FOXN1 may be implicated as a cofactor in the development of vital systems required for a proper fetus development. This would explain the high mortality rate during the first trimester of pregnancy reported among consanguineous carriers of the mutation [74], which is not justified by the SCID *per se* due to the maternal protection until the third month after birth.

CONCLUSIONS

The simultaneous occurrence of severe functional T-cell immunodeficiency and skin abnormalities associated with *FOXN1* alterations indicates that the factor exerts a critical role in the development and homeostasis of these epithelia and suggests shared functions of the gene in both thymus and skin epithelium. Evidence is available showing that a co-culture containing skin-derived cells and hematopoietic precursor cells (HPCs), reconfigured in 3D arrangement, expressing high levels of FOXN1, reproduce a thymus organoid able to generate mature and functional T cells from precursor cells even in the absence of thymic cells [77]. This central role of FOXN1 in ensuring a proper T-cell ontogeny is also indirectly sustained by the presence of functional T cells in DGS but not in Nude/SCID.

Despite the significant progress to date made, the detailed mechanism by which FOXN1 controls the T-cell differentiation process through intercellular cross-talk still remains to be clarified. Most of the target genes regulated by FOXN1 are sill undefined, also due to the technical difficulties to isolate intact TECs at different developmental stages.

Additional knowledge in this field would be very helpful in conclusively defining the role of FOXN1 in the biological process, in clarifying the intimate mechanisms of FOXN1 action and in the development of novel therapeutic strategies for congenital disorders of immune system.

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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FOXN1: a master regulator gene of thymic epithelial development program

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T cell ontogeny is a sophisticated process, which takes place within the thymus through a series of well-defined discrete stages. The process requires a proper lympho-stromal interaction. In particular, cortical and medullary thymic epithelial cells (cTECs, mTECs) drive T cell differentiation, education, and selection processes, while the thymocyte-dependent signals allow thymic epithelial cells (TECs) to maturate and provide an appropriate thymic microenvironment. Alterations in genes implicated in thymus organogenesis, including *Tbx1*, *Pax1*, *Pax3*, *Pax9*, *Hoxa3*, *Eya1*, and *Six1*, affect this well-orchestrated process, leading to disruption of thymic architecture. Of note, in both human and mice, the primordial TECs are yet unable to fully support T cell development and only after the transcriptional activation of the *Forkhead-box n1* (*FOXN1*) gene in the thymic epithelium this essential function is acquired. *FOXN1* is a master regulator in the TEC lineage specification in that it downstream promotes transcription of genes, which, in turn, regulate TECs differentiation. In particular, *FOXN1* mainly regulates TEC patterning in the fetal stage and TEC homeostasis in the post-natal thymus. An inborn null mutation in *FOXN1* leads to Nude/severe combined immunodeficiency (SCID) phenotype in mouse, rat, and humans. In *Foxn1*$^{-/-}$ nude animals, initial formation of the primordial organ is arrested and the primordium is not colonized by hematopoietic precursors, causing a severe primary T cell immunodeficiency. In humans, the Nude/SCID phenotype is characterized by congenital alopecia of the scalp, eyebrows, and eyelashes, nail dystrophy, and a severe T cell immunodeficiency, inherited as an autosomal recessive disorder. Aim of this review is to summarize all the scientific information so far available to better characterize the pivotal role of the master regulator FOXN1 transcription factor in the TEC lineage specifications and functionality.

Keywords: Foxn1 gene, TECs, thymus gland, immunodeficiency, Nude/SCID

INTRODUCTION

The thymus is the primary lymphoid organ with the unique function to produce and to maintain the pool of mature and functional T cells. This process is strictly dependent on specialized functions of thymic stromal cells (TSCs) and requires the thymus peculiar tridimensional (3D) architecture, which allows a proper intercellular cross talk (1). For a long time, the difficulty in the isolation and characterization of the thymic cellular components has limited studies on the peculiar role of individual stromal components. Novel experimental tools, including stromal cell isolation by phenotype-based cell sorting (2), dissociation and reaggregation of stromal cell subsets (3, 4), or global gene expression analysis and the evaluation of the pattern of self-antigen expression within the individual thymic epithelial cells (TECs) subset (5), allowed to acquire important knowledge on the cellular and molecular basis of thymus organogenesis and TECs functionality.

The recent discovery of disease models associated to genetic alterations of molecules implicated in thymus specification and TECs differentiation, provided new and conclusive insights regarding the pathways, the genes, and the molecular mechanism governing these processes and stromal functionality.

THE THYMUS ARCHITECTURE: REQUIREMENT OF A 3D STRUCTURE FOR A PROPER LYMPHO-EPITHELIAL CROSSTALK

The thymus provides the microenvironment essential for the development of T cells. T cell progenitors originate in the bone marrow, enter into the thymus (6, 7) and, through a series of well defined and coordinated developmental stages, differentiate, undergo selection process, and mature into functional T cells. The steps in this process are tightly regulated through a complex network of transcriptional events, specific receptor-ligand interactions, and sensitization to trophic factors, which mediate the homing, proliferation, survival, and differentiation of developing T cells (1, 8, 9).

The thymus is organized in two lobes, which are already present in mice at 21 days of thymic organogenesis and is completely organized at 1 month of post-natal life. The lobes are divided in three areas: a cortical and the dark cortical area, with a high number of lymphoid cells and epithelial cells, cortical thymic epithelial cells (cTECs); a light medullary area with a low number of mature T cells, named medullary TECs (mTECs), Hassall's bodies (HB), macrophages, dendritic cells (DCs), B lymphocytes, and

rarely myoid cells. Eventually, there is a transitional area, named cortico-medullary junction (CMJ), characterized by abundant blood vessels (10).

The unique function of the thymus in the establishment and maintenance of the T cell pool is intimately linked to this peculiar thymus architecture and to the specialized functions of the TSCs.

LYMPHO-EPITHELIAL CROSS-TALK REQUIRED FOR THYMOCYTE AND TECs DIFFERENTIATION

An important feature of the thymic microenvironment is its 3D organization, consisting of an ordered architecture of TSCs, that represents a heterogeneous mixture of distinct cell types, including cTECs, mTECs, fibroblasts, endothelial cells, DCs, and macrophages (11). Among these stromal elements, TECs are the most abundant cell types, which form a delicate 3D cellular network spanning throughout both the thymic cortex and the medulla. The requirement for the 3D-supporting stroma appears to be unique to the T cell development, as the *in vitro* differentiation program of other hematopoietic lineages, including B and NK cells, does not require a 3D structure (12).

Thymocyte development is not a cell-autonomous process, and the transition to the next stage in development relies on the proper interaction of HSCs with thymic stroma. The 3D configuration of the thymus maximizes this interaction, allowing intercellular cross-talk integral to the development of both T cells and TSCs (13). Paralleling the T cell precursor proliferation and differentiation program, immature TECs undergo a developmental sequence, resulting in the establishment of mature cTECs and mTECs organized in this 3D network. Several studies on mutant mice with an abnormal organization of thymic epithelium substantiated the concept that a reciprocal signaling between thymocytes and TSCs is required, not only for the production of mature T cells but also for the development and organization of the thymic microenvironment in a bi-directional fashion (14, 15). Mice showing a blockage of the T cell development process, in the absence of T cell receptor (TCR)-expressing cells, have a defective organization of the thymic medulla, as well (16, 17). Of note, under this condition, thymic medullary organization can be restored by the addition of mature T cells, which follows stem cell transplantation (17, 18). In adult CD3etg26 mice, lacking intra-thymic T cell precursors, a severe alteration of the cortical thymic architecture has been documented (19), even though a restoration of the architecture and TEC development in these mice can occur. Recently, the injection of either fetal or adult T-committed precursors into adult CD3etg26 mice leads to the reconstitution of thymic microenvironment, as indicated by thymocyte differentiation, organization of functional cortical and medullary areas, and generation of $Foxp3^+$ T_{reg} and $Aire^+$ mTECs (20). These data suggest that adult TECs maintain the receptivity to cross talk with thymocytes despite a prolonged absence of T cell precursors. Moreover, the absence of both thymocytes and of the 3D framework may result in changes of the keratin genes expression, thus inducing the cTECs and mTECs to undergo a de-differentiation process and to reacquire the precursor $K5^+K8^+$ cellular phenotype. Taken together, these findings suggest that signals from early $CD4^-CD8^-$ DN T cell precursors and/or their immediate progeny provide necessary signals to promote the formation of the thymic cortex, while, later in ontogeny, the differentiation of TECs into a medullary phenotype are clearly dependent on the presence of $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive (SP) thymocytes (21–23). However, the precise molecular nature of the signals provided by developing thymocytes, which lead to the generation of the thymic stromal compartment are still incompletely defined.

Eventually, a better understanding of the developmental process through which a normal thymus structure is built, is essential for a better comprehension of the intimate mechanisms which take place within the thymus to promote the T cell development *in vivo*. This knowledge may also be useful in designing future therapeutic strategies, as alterations of the thymus structure and function may result in serious health consequences, including immunodeficiency or autoimmunity.

mTECS AND cTECS ARE SPECIALIZED CELLS PLAYING A DIFFERENT ROLE IN THE T CELL EDUCATION PROCESS

T cell ontogeny is a sophisticated process, which takes place through discrete stages during which developing thymocytes dynamically relocate in different thymic areas, following a cortico-medullary gradient.

The initial colonization of the thymus anlagen by migrant lymphoid progenitors occurs at an early stage, embryonic day 11.5 (E11.5) in mice and 8 week of gestation in humans (24, 25). Studies documented that chemokines CC ligand (CCL)21 and CCL25 play a major role in the early stage of fetal thymus colonization (26, 27). Indeed, mice deficient for these chemokines or for the cognate receptors, showed a significant reduction in the number of thymocytes compared to normal mice (28). In post-natal thymus, lymphoid progenitor cells through their cell surface adhesion molecules, such as platelet-selectin glycoprotein ligand 1, interact with P-selectin, expressed on the TECs, and thanks to this interaction they are allowed to migrate from the blood into the thymic parenchyma, in correspondence of the area around the CMJ [**Figure 1**; (29)].

Entered thymocytes started to intensely proliferate and to acquire T cell hallmarks. In this phase, T cell proliferation and differentiation are triggered by a potent combination of signals provided by cTECs. Delta-like 4 (DL4), which is an essential, non-redundant ligand for Notch1 during thymic T cell development, and IL-7 are critically involved in the activation of signaling pathways, leading to the proliferation and migration of thymocytes (30–32). In particular, these intra-thymic ligands induce the development of DN $CD25^+$ cells, which migrate toward the subcapsular region of thymic cortex (33). Several chemokine receptors have been suggested to guide the migration of immature thymocytes, such as CXCR4, CCR7, and CCR9 [**Figure 1**; (34)]. In the thymic cortex DN thymocytes begin V(D)J rearrangement of their TCRβ gene. Successfully rearranged TCRβ protein, assembled with the pre-TCRα chains, forms the pre-TCR complex. Membrane expression of pre-TCR complex, along with the Delta-Notch interaction, provides the signal necessary to induce the expression of the co-receptors CD4 and CD8, as well as V-J rearrangement of the TCRα genomic region. Subsequently, DP thymocytes with a functional TCR-αβ receptor are generated [**Figure 1**; (35)].

Thymic cortex is also the area where takes place the positive selection of DP thymocytes. Positive selection is the process by

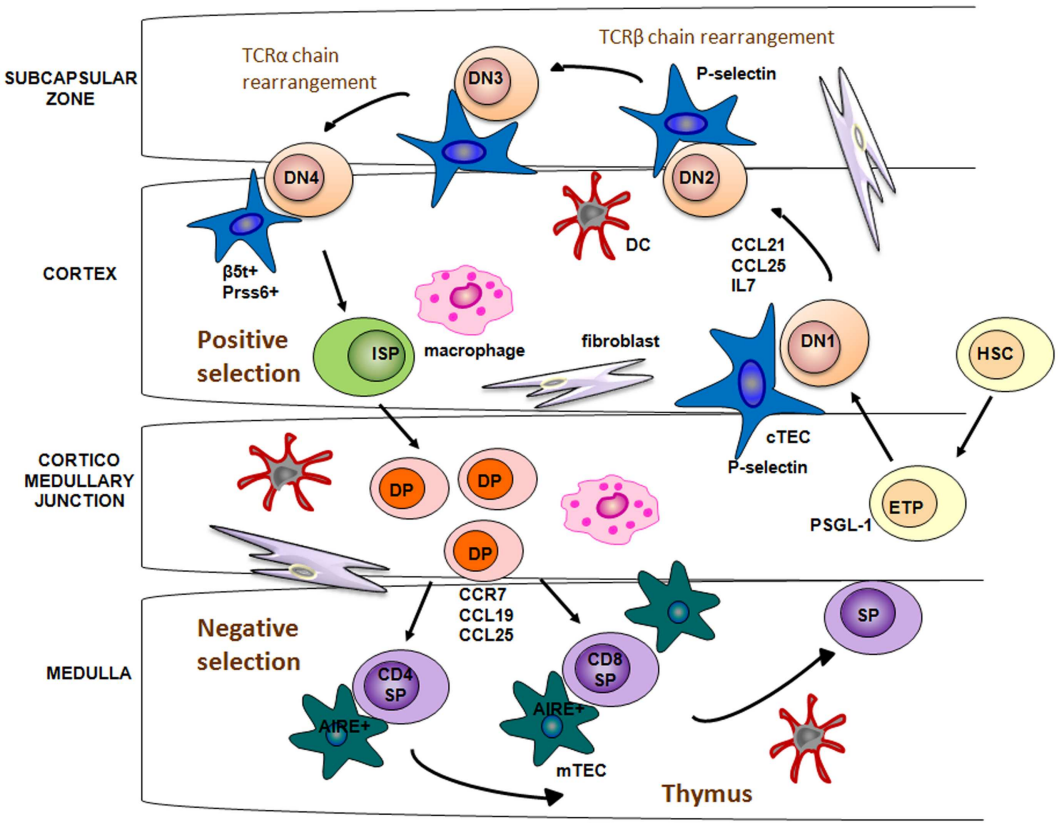

FIGURE 1 | Lympho-stromal interactions and T cell development. Bone marrow HSCs enter into the thymus through CMJ, a process mediated by CCL12 and CCL25 in the embryonic thymus and by the interaction between P-selectin and its cognate ligand PSGL-1 in adult thymus. Stimulation by IL-7 allows the relocation of DN thymocytes from the cortex to the subcapsular region. DP thymocytes bearing TCR and capable of binding to self-MHC ligands are positively selected. This process is regulated by Pssr6 and b5t, which are expressed in cTECs. Developing thymocytes are relocated from cortex to the medulla by chemotactic attraction between CCR7 and the ligands CCL19/CCL21, expressed on the mTECs. Into medulla, self-reactive thymocytes are deleted through the negative selection, a process mediated by dendritic cells and Aire-expressing mTECs.

which developing thymocytes, that recognize and bind with mild avidity peptide-major histocompatibility complex on cTECs surface, get a rescue signal through their TCR and are allowed to further maturate to the $CD4^+CD8^-$ or $CD4^-CD8^+$ SP stage. Only a small fraction (1–5%) of DP cells survive to positive selection. By contrast, the majority of DP cells, that bind with too low affinity to MHC complex, are programed to undergo death by neglect (36, 37).

Cortical thymic epithelial cells have a crucial role in the positive selection process of T cells within thymus cortex (38). Recent studies have found that cTECs exclusively express a specific form of proteasome, referred as thymoproteasome, which contains a peculiar catalytic subunit, the β5-thymus (β5t) (39). β5t subunits exhibit an unique peptidase activity, compared to other β5 subunits found in common immunoproteasome, which leads to the production of a set of self-peptides with a high affinity for class I MHC molecules (40). Moreover, β5t-deficient mice show a severe decrease in the number of $CD8^+$ SP thymocytes, but no alteration in the $CD4^+$ number or in the thymic architecture. In addition, the small fraction of $CD8^+$ T cells, positively selected by β5t-deficient cTECs, show altered immune responses toward several stimuli. Taken together these results suggest that the thymoproteasome is essential for the production of self antigens involved in the positive selection of functional $CD4^-CD8^+$ T cells (41).

As for the positive selection of $CD4^+$ T cells, two other proteins predominantly expressed in cTECs, the lysosomal protease Prss16 and Cathepsin L, have been demonstrated to be essential to generate an immunocompetent repertoire of $CD4^+CD8^-$ T cells [**Figure 1**; (42, 43)].

TCR engagement by peptide-MHC complex also triggers the expression of the chemokine receptor CCR7 in positively selected thymocytes. Thanks to the chemotactic attraction between CCR7 and its ligands, CCL19 and CCL21, expressed on the mTECs, developing thymocytes are relocated from cortex to the medulla [**Figure 1**; (44, 45)].

In order to create a repertoire of mature T cells able to recognize foreign antigens and, at the meantime, to ignore self antigens, SP thymocytes have to undergo the negative selection process in the thymic medulla. Both mTECs and DCs, play a pivotal role in this last stage of thymocyte development, which is critical to establish the central tolerance and, eventually, to prevent autoimmunity. In contrast to cTECs, mTECs are characterized by a high expression

of clustered tissue-restricted autoantigens (TSAs), the so called promiscuous gene expression (46). To date, the autoimmune regulator (AIRE) transcription factor represents the only molecule, so far identified, which contributes to the mTECs function and, in particular, to the molecular regulation of the promiscuous gene expression [**Figure 1**; (47)]. However, not all TSAs are regulated in an AIRE-dependent manner, suggesting that other molecular mechanisms, such as epigenetic mechanisms, may be involved in mTECs function regulation. TSAs associated with class II MHC molecules are presented directly by mTECs or indirectly by DCs to developing thymocytes (48). T cells which recognize with a high avidity self antigens are deleted. Remarkably, only a few number of mTECs express a given TSAs (about 50–500 per thymus), and lead to apoptosis by negative selection of a few thymocytes (37, 49, 50). A possible explanation is that the high motility of thymocytes within the thymic medulla during a period of 4–5 days, allows each of them to interact with mTECs (51). DCs play a similar role in the negative selection process. They are attracted in the thymic medulla by the chemochine XCL1 (lymphotactin), produced by mTECs in an AIRE-dependent manner. Differently from mTECs, DCs are not able to produce TSAs and the TSAs expressed mostly derive from the phagocytosis of apoptotic mTECs (52, 53). mTECs and DCs not only contribute to the establishment of central tolerance through the deletion of self-reactive T cells, but, also, through the generation of regulatory T cells (T_{regs}) (54, 55, 65, 153), which act in the periphery by suppressing autoreactive T cells, which have escaped to the process of the central tolerance.

A body of evidence documents that the expression of an autoreactive TCR leads to the entry of the thymocyte into the T_{reg} lineage. T_{regs}, that are about 5–10% of peripheral T cells $CD4^+$, constitutively express the CD25 molecule and share several immunological features, in humans and mice (56, 57). These cells specifically express the transcription factor FOXP3 (Foxp3 in mice) that plays a pivotal role in T_{regs} differentiation and function (58). The Foxp3 promoter region and the conserved non-coding sequence 2 (CNS2) (known as TSDR, the T_{reg}-specific-demethylated-region) are fully methylated in immature thymocytes (59, 155). At the beginning of T_{reg} development, an appropriate TCR/CD28 signal is needed to make available the *Foxp3* promoter through shift of the Protein Inhibitors of Activated STAT 1 (PIAS1), a signal cascade, which results in the NF-κB-mediated transcription of genes playing a role in T_{reg} differentiation (60, 61).

THYMIC FORMATION: NEW INSIGHTS IN EPITHELIAL LINEAGES SPECIFICATION

In the mouse, mTECs and cTECs originated from the third pharyngeal pouch endoderm and the thymus anlage are located next to that of the parathyroid. The expression of Forkhead-box transcription factor n1 (Foxn1) approximately at E11.5 is crucial for the subsequent epithelial differentiation, since in its absence, the colonization of the anlage by T cell progenitors from the bone marrow fails (62) and the subsequent T cell development and TECs formation is aborted, resulting in a severe immunodeficiency (63, 64, 66, 154).

The maturation process of TECs during thymic organogenesis could be divided in two genetic phases. The first stage is independent from the *Foxn1* expression and consists in the induction and outgrowth of the thymic epithelial anlage from the third pharyngeal pouch, through the expression of genes including the *Eya1* and *Six* (67), *Hoxa3* (68), and *Tbx1* (69, 70). During the second genetic phase, epithelial patterning and differentiation take place and the *Foxn1* expression drives the immature epithelial cells to differentiate into functional cTECs and mTECs (71).

FOXN1-INDIPENDENT GENETIC STAGE OF TEC DIFFERENTIATION

In the first phase of the thymus organogenesis an interaction between epithelial and mesenchymal cells occurs, while at the later phase lympho-epithelial interaction predominates (72). In mice, at about E10.5 the mesenchymal cells are able to respond to the endodermic signals, which induce the development of the primordial thymic epithelium (73, 74). Subsequently, at about E12.5, the thymic rudiment is colonized by progenitors come from the fetal liver, thus resulting in a tight epithelial-thymocyte interaction within the mesenchymal derived capsule. This thymic rudiment contains the $EpCam^+Plet1^+$ epithelial population (72, 75), which includes a common thymic epithelial precursor (TEPC), from which both cTECs and mTECs will be subsequently generated (72, 76).

Through studies on animal models carrying molecular alterations of distinct genes, the key role of several transcription factors involved in the thymus organogenesis and TEC-sublineage specification process, have thus far been identified (77). In particular, several genes, including *Tbx1* (69, 70), *Pax1*, *Pax3*, *Pax9* (78–80), *Hoxa3* (68), *Eya1*, and *Six1* (67) have been shown to play a central role in the thymus ontogeny. Indeed, their molecular alteration affects this well-orchestrated process, leading to disruption of the thymic architecture. Abnormalities of the paired box (Pax) family transcription factors Pax1 or Pax9 result in a blockage of the thymus organogenesis (79, 81). Mutations in the Hox transcription factor family member, Hoxa3, expressed on both thymic epithelium and mesenchymal cells, result in athymia (68). Furthermore, the homozygous loss of *Tbx1*, related to the DiGeorge syndrome phenotype, leads to thymic a/hypoplasia in humans (69, 82), while mice heterozygous for a null allele of *Tbx1* show a mild phenotype without thymus anomalies (83). Therefore, the expression of *Tbx1* both in the pharyngeal core mesoderm and in the pharyngeal endoderm is required for a proper thymus development. However, it remains to be elucidated whether the expression of *Tbx1* in the TECs occurs and whether the gene participates in the TECs development (4).

FOXN1-DEPENDENT GENETIC STAGE OF TEC DIFFERENTIATION

In both humans and mice, the primordial TECs are yet unable to fully support T cell development and only after the transcriptional activation of the *FOXN1* gene in the thymic epithelium this essential function is acquired. *FOXN1* is a master regulator in the TEC lineage development in that it promotes down-stream the transcription of genes implicated in the thymus organogenesis and TECs full differentiation.

Forkhead-box n1 transcription factor belongs to the FOX transcription factor family implicated in a variety of biochemical and cellular processes, including development, metabolism, aging, and cancer (84, 85). During the post-natal life, *Foxn1* is selectively

expressed only in thymic and skin epithelia, where it regulates the expression of several molecular targets to maintain the balance between growth and differentiation (86, 87). The signals required for *FOXN1* expression, and its activity, are still unclear, even though the wingless (Wnt) proteins (88) and bone morphogenetic protein (BMP) signaling have been shown to regulate *FOXN1* expression (89). Even though the complete pattern of *FOXN1* expression over the time and its role are not yet completely defined, studies on mouse and human model of gene alterations enormously helped unravel important issues on its role. Mutations in *Foxn1* gene lead to alymphoid cystic thymic dysgenesis due to a defective TECs differentiation process (63, 90). In both mice and humans *FOXN1* abnormalities lead to a hairless phenotype (87, 154).

In the *Foxn1*-dependent step of thymus organogenesis, precursor epithelial cells differentiate into mature and functional cTECs and mTECs from the same bi-potential TEC progenitor (4, 72, 76). It has been reported, that *Foxn1* is differentially expressed during the TE-lineage specification, since it is expressed in all TECs during the pre-natal life, but not in all TECs postnatally, indicating that the gene is highly developmentally regulated. There is a body of evidence documenting different effects of *Foxn1* expression in mTEC and cTECs. Particularly, studies on K5- and K18-CreERT-mediated *Foxn1*-deleted mouse models suggested that during the post-natal life, the loss of *Foxn1* affected mTECs, characterized by the expression of K5 and K14 keratins type. Conversely, the loss of *Foxn1* did not affect cTECs, which express the keratins K8 and K18 (91, 92). Taken together, these data suggest that cTECs and mTECs are not equally *Foxn1*-dependent in the post-natal life.

Recent reports highlighted a central role for *Foxn1* in TECs homeostasis in the adult thymus and its necessary role for the functionality and survival of adult TEC progenitors (92), expressing $K5^+$ and $K14^+$ markers. This role in adult thymus seems to be exerted in cooperation with other stem cell-related genes, such as *p63*. Of note, the transcription factor p63, encoding for multiple isoforms (93), plays a pivotal for the development of stratified epithelia of several tissues, such as epidermis, breast, prostate, and thymus (94). In the thymus, the p63 protein drives the proliferation of epithelial progenitor cells (94, 95). Therefore, it has been hypothesized that *p63* and *Foxn1* could act synergistically through the formation of a *p63-Foxn1* regulatory axis aimed at regulating TECs homeostasis. However, the molecular mechanism through which the proliferation regulator *p63* and differentiation regulator *Foxn1* collaborate in this axis are still unclear.

FOXN1-MEDIATED GENE EXPRESSION FOR TEC DIFFERENTIATION

Forkhead-box n1 is directly or indirectly implicated in the transcriptional regulation of a panel of genes involved in thymus development and function.

Pax1 is a key regulator of TEC differentiation/survival balance. *Pax1* is expressed in the third pharyngeal pouch from E9.5 during the thymus ontogeny, while in the post-natal thymus only in cTEC (96). Even though the regulation of *Pax1* is still unclear, from E11.0 its expression requires *Hoxa3* (68). Of note, the loss of *Hoxa3* impairs the intrinsic ability of the neural crest cell population to differentiate and/or to lead to the differentiation of the tissues of pharyngeal arch and pouch. Indeed, in *Hoxa3* mutant mice the thymus is absent and thyroid hypoplasia has been documented (68). Moreover, the first step of thymus development is the expansion of mesenchymal neural crest in the posterior part of the third pharyngeal pouch. Prior to this event, in the *Hoxa3* mutant embryos a marked reduction in *Pax1* expression has been shown. Similarly, *Pax1* mutant mice also show thymic hypoplasia, suggesting a role for *Hoxa3* in maintaining *Pax1* expression in these cells (68). In the thymic primordium, *Pax1* expression is under the control of *Foxn1* (71). This finding indicates that *Foxn1* and *Hoxa3* are both involved in the network of molecular signals that regulates *Pax1* expression, thus demonstrating the existence of a molecular and/or functional interaction between *Hoxa3* and *Foxn1* [**Figure 2**; (71)]. In keeping with this, $Hoxa3^{+/-}Pax1^{-/-}$ compound mutant mice display a few phenotypic hallmarks of the $Foxn1^{R/R}$ mouse model, which expresses low-dose of *Foxn1*, such as hypomorphic post-natal thymus, and reduced levels of MHC class II expression on the TECs surface (80). These data suggest two alternative hypothesis: *Hoxa3* may regulate *Foxn1*, which, in turn, regulates *Pax1* expression in the thymic primordium, in a *Foxn1*-dependent manner, or *Hoxa3* and *Foxn1* induce *Pax1* expression in the third pharyngeal pouch and in early thymus primordium.

It has also been shown that *Foxn1* regulates the expression of *CCL25* and *Dll4* (**Figure 2**). These genes play a pivotal role in the thymocyte development, since *CCL25* regulates the colonization of the fetal thymus (97), while the Notch ligand *Dll4* is involved in the commitment of hematopoietic progenitors to the T cell lineage (30). In both early fetal TEC and in the post-natal thymus, *Dll4* expression is directly related to the *Foxn1* expression (71). Furthermore, these molecules are absent in the *Foxn1* null thymus, even though there is evidence indicating that their expression may occur in a *Foxn1*-independent manner in TECs (98, 99). Eventually, in a recent report it has been shown that *Foxn1* is upstream of dll4a and ccl25a expression in *medaka fish*, thus confirming the relationship with this transcription factor (100).

THE HUMAN NUDE/SCID PHENOTYPE: A MODEL OF THYMIC MICROENVIRONMENT DISRUPTION AND FAILURE OF THE T CELL DEVELOPMENT

The Nude/severe combined immunodeficiency (SCID) phenotype represents the prototype of thymic architecture disruption due to alterations of the *FOXN1*, which is the master regulator of TE-lineage specification (71).

In humans, as in mice and rats, mutations in the "nude" *Foxn1* gene induce the hairless phenotype, associated with a rudimentary thymus gland (T cell related primary immunodeficiency). The human Nude/SCID phenotype (MIM 601705; Pignata Guarino Syndrome) was first identified in 1996, after more than 30 years from the initial mouse description, in two sisters originated from a small community with a high grade of inbreeding, who showed congenital alopecia of the scalp, eyebrows, and eyelashes, nail dystrophy, and a severe T cell immunodeficiency, inherited as an autosomal recessive disorder (154). This phenotype was associated with a C792T transition in the *FOXN1* gene, which resulted in the nonsense mutation R255X in the exon 4 (formerly exon 5), with a complete absence of a functional protein similar to the previously described rat and mouse *Foxn1* mutations (101–103).

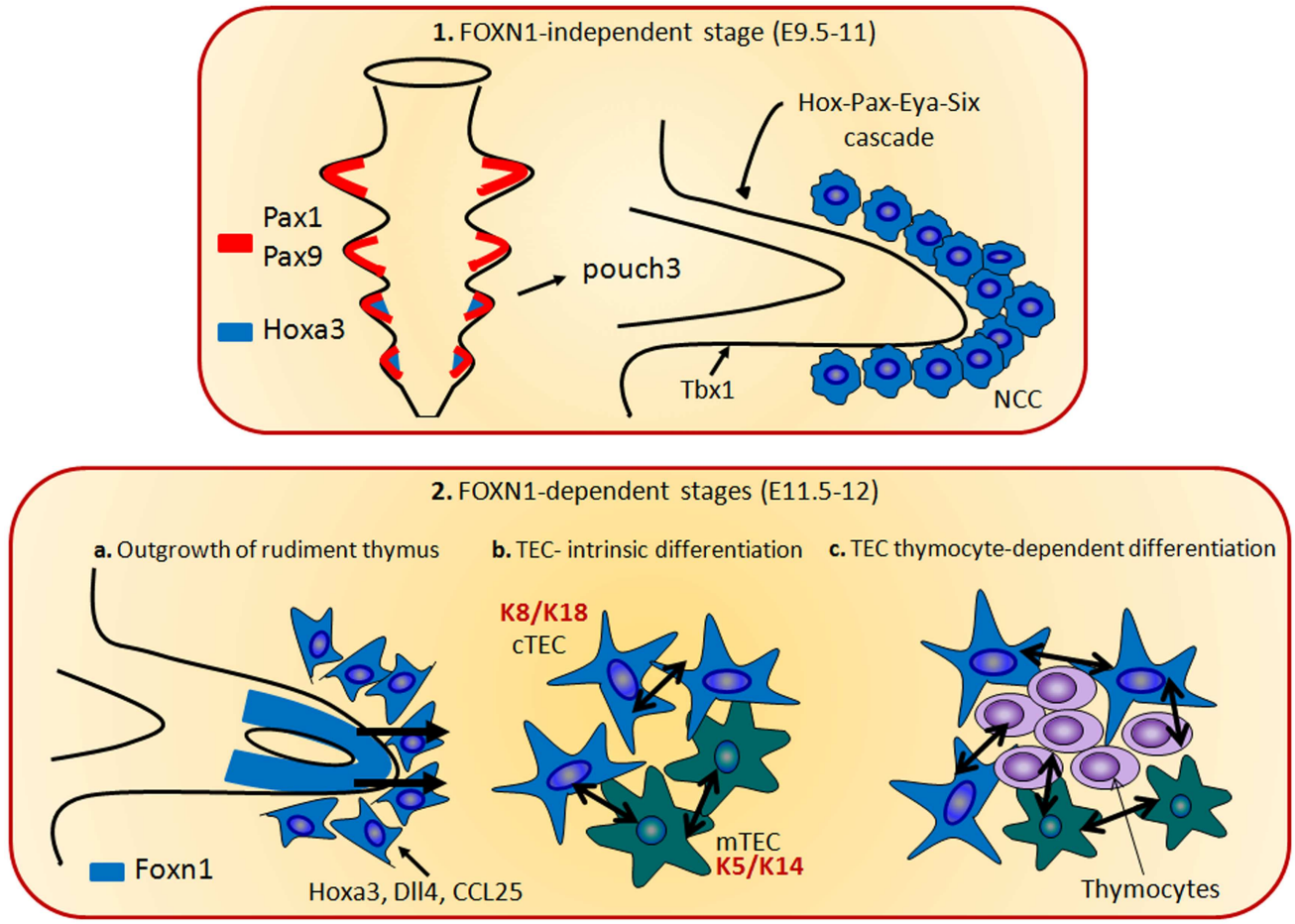

FIGURE 2 | The thymus organogenesis. The thymus organogenesis is characterized by two genetic phases. The first stage is independent from the *Foxn1* expression (1) and consists in the induction and outgrowth of the thymic epithelial anlage from the third pharyngeal pouch, through the expression of genes including the *Eya1*, *Six*, *Hoxa3*, and *Tbx1*. *Hoxa3* and *Eya1* are also required in neural crest cells (NCCs). In the phase 2, *Foxn1* regulates the expression of *CCL25*, *Dll4*, and *Hoxa3*, necessary for the thymocytes and TECs differentiation. During this step, cTECs (expressing K8 and K18 keratin type) and mTECs (expressing K5 and K14 keratin type) originate from the same bi-potential TEC progenitor. The crosstalk between TECs and developing thymocytes is required to generate fully mature TECs and functional T cells.

In the absence of *Foxn1* expression, thymic development is halted at a rudimentary stage. As a consequence, in the affected patients the thymic lobe is still present but intra-thymic lymphopoiesis is completely blocked (63, 104) leading to severe primary T cell immunodeficiency (105–107) and to death in early childhood from severe infections (105, 108–112, 154). *Foxn1* is also involved in morphogenesis and maintenance of the 3D thymic micro-structure, which is necessary for a fully functional thymus (113, 114). In fact, evidence is available that in an *in vitro* 2D culture system consisting of a monolayer of mouse bone marrow stromal OP9 cells it is possible to generate mature T cells, only if these cells are transduced with the Notch ligand Delta-like 1 (OP9-DL1) (115, 116), whose pathway exerts a pivotal and necessary role in promoting the induction of T cell-lineage commitment (117–119). Of note, in all these co-culture systems, the stromal cells are enforced to overexpress Notch ligands, and their expression by TECs seems to be maintained only in a 3D thymus structure (120). In human Nude/SCID, the T cell defect is characterized by the absence of proliferative response to the common mitogens and a severe blockage of the T cell differentiation (154). Recent studies revealed the presence of some circulating T cells of non-maternal origin in patients carrying alterations of *FOXN1* gene. These cells have been shown to be predominantly double-negative αβ T cells ($CD3^+CD4^-CD8^-$, DN) and to exhibit a regulatory like T cell phenotype ($FoxP3^+$). This finding raised important issues regarding the site of differentiation of these cells. One hypothesis is the persistence of a thymic rudiment, which allows a partial T cell development (109). Alternatively, a T cell differentiation, even though partial and ineffective to result in a productive immunity, could occur at an extra-thymic site. In both pre-natal and post-natal life, the TCRBV spectratype repertoire in Nude/SCID patients is oligoclonal, thus confirming the immaturity of the process and, at the same time, that developmental events do take place at some extent (111, 112).

For many years, the human counterpart of the nude mouse phenotype has been erroneously considered the DiGeorge syndrome,

which occurs spontaneously and is mainly characterized by thymic hypo/aplasia and a mild T cell defect. However, several lines of evidence argue against the analogy between these two disorders. In fact, the DiGeorge syndrome is often associated with neonatal tetany and major anomalies of great vessels. These defects are due to malformation of the parathyroid and heart, derived from a major embryologic defect in the third and fourth pharyngeal pouch from which the thymus primordium emerges. In addition, in this syndrome hairlessness is missing and gross abnormalities of skin annexa are not found. Children with DiGeorge syndrome may also have lymphopenia, with a mild reduction of T cells, that are however usually responsive to common mitogens.

In Nude/SCID patients, skin is tighter than usual and is characterized by basal hyperplasia and dysmaturity. Alopecia is primitive in nature, in that it can be observed at birth and persists after bone marrow transplantation, thus ruling out the acquired nature of the disorder. In keeping with this, in athymic mice, completely lacking body hair, restoration of the thymus did not lead to hair growth, indicating a direct participation of FOXN1 to hair follicle development (87). The most frequent phenotypic alteration affecting the nails is koilonychia ("spoon nail"), characterized by a concave surface and raised edges of the nail plate, associated with significant thinning of the plate itself; canaliform dystrophy and a transverse groove of the nail plate (Beau line) may also be observed (121). However, the most specific phenotypic alteration is leukonychia, characterized by a typical arciform pattern resembling a half-moon and involving the proximal part of the nail plate. These alterations of digits and nails were also reported in a few strains of nude mice. Of note, nail dystrophy has also been observed in heterozygous subjects carrying *FOXN1* alterations (121). *FOXN1* is known to be selectively expressed in the nail matrix, where the nail plate originates, thus confirming that this transcription factor is involved in the maturation process of nails and suggesting nail dystrophy as an indicative sign of heterozygosity for this molecular alteration (121).

Autoptical study of a fetus homozygous for R255X mutation revealed multiple-site neural tube defects, including anencephaly and spina bifida. This finding may help explaining the high rate of mortality *in utero* observed in the population where the first patients were identified (105). Intriguingly, the other forms of SCID become clinically evident only during the postnatal life, when the protection of the newborn transferred from the mother immune system declines. This observation, suggests that other causes different from immunodeficiency, are responsible for the high rate of mortality *in utero* and led to consider the Nude/SCID mutation and anencephaly causally related. Of note, in a recent study, the mouse Foxn1 gene was found to be expressed also in epithelial cells of the developing choroids plexus, a structure filling the lateral, third and fourth ventricles of the embryonic brain (105). Moreover abnormality in the development of corpus callosum were also found in another FOXN1 mutated fetus even in the absence of anencephaly, indicating that the transcription factor may play a role as a co-factor in the brain ontogeny (105).

Altogether these findings suggest that FOXN1 may also be implicated as co-factor in the development of vital systems required for a proper fetus development, thus explaining the mortality in the first trimester in fetuses carrying the genetic alterations, which is not justified by the SCID *per se*.

FOXN1 MUTATION PREVENTS THE PRE-NATAL T CELL DEVELOPMENT IN HUMANS

It is now clear that FOXN1 acts as a transcription factor implicated in the differentiation of thymic and skin epithelial cells, even though many of its molecular targets still remain to be discovered. Most of the knowledge so far available has been achieved in humans in the post-natal life, while little is known about FOXN1 role during the pre-natal life.

Of note, other FOX family members, including *Foxq1* and *Foxm1b*, are important during embryogenesis, being involved in a variety of biological processes (122). Approximately 50% of *Foxq1*$^{-/-}$ murine embryos die *in utero*, thus suggesting the requirement of this gene during embryogenesis (123). Similarly, *Foxm1b* is important during liver regeneration (124).

Studies on thymus organogenesis revealed that *Foxn1* is expressed in all TECs during fetal stages. Of note, *Foxn1*$^{-/-}$ mice showed undifferentiated TECs responsible for a blockage of thymopoiesis and severe immunodeficiency (125). Recently, the identification of a human *FOXN1*$^{-/-}$ fetus gave the unique opportunity to study in humans the T cell development *in utero*, in the absence of a functional thymus. Vigliano et al. documented a total blockage of the CD4^{+} T cell maturation and a severe impairment of CD8^{+} cells, with an apparent bias toward TCRγδ^{+} cells (112). In this case in the congenital absence of the thymus was due to R255X missense mutation in the *FOXN1* gene. In particular, it has been reported that in the absence of FOXN1 a few not functional CD8^{+} cells, mostly bearing TCRγδ in the absence of CD3, presumably of extra-thymic origin could develop in both humans and mice (126–128). Further analysis of the fetal RNA, performed to evaluate the variable-domain β-chain (Vβ) families' usage among T lymphocytes, revealed that the generation of TCR diversity occurred at some extent in the *FOXN1*$^{-/-}$ fetus, but was abnormal. Thus, these data provided a further evidence of the crucial role for FOXN1 in the early pre-natal stages of T cell development and not in the B and NK-cell differentiation, these populations being normally present in the Nude/SCID fetus (112). A similar impairment of the T cell differentiation with a selective blockage of CD4 differentiation but not of CD8, was detected in murine models characterized by the absence of the nuclear high-mobility group (HMG) box protein TOX (107).

The identification of a limited number of CD8^{+} cells bearing the TCRγδ suggests that this cell population may develop at extra-thymic sites in a *FOXN1*-independent manner, even though they are unable to sustain a productive immune response into the periphery. Indeed, evidence exists indicating that T cells may also differentiate at extra-thymic sites, as intestine and liver (129–133). Of note, the majority of thymus-derived T lymphocytes bears the αβ chains of TCR and a few of them express the γδ heterodimer (134), while the T cell pool developed outside the thymus is characterized by a higher proportion of TCRγδ^{+} T cells expressing the CD8αα homodimer, instead of the CD8αβ (135, 136). Moreover, also DN T cells (CD3^{+}CD4^{-}CD8^{-}) and lymphocytes expressing CD7 and CD2 in the absence of CD3 (CD2^{+}CD3^{-}CD7^{+}) are generally considered of extra-thymic origin (135–137).

In spite of the well documented knowledge on the role of the primary lymphoid organ to foster T cell development, some still unsolved issues in human athymic conditions indicate that an in-depth information of the overall process is still to be achieved and, in particular, the involvement of different tissues in T cell ontogeny must be definitively clarified. Since FOXN1 is selectively expressed in the thymus and skin, one possibility to explain the presence of the few non-functional $CD8^+TCR\gamma\delta^+$ cells in Nude/SCID fetus is that skin epithelial cells could play a partial role in T cell ontogeny, as already shown in *in vitro* models (138, 139).

THYMUS TRANSPLANTATION: A PROMISING TREATMENT TO ATHYMIC DISORDERS

Forkhead-box n1 deficiency is a very rare immunodeficiency with unfortunately poor chance of curative treatments. Recently, thymus transplantation has emerged as a promising treatment for children affected with congenital athymia (140–143), as that observed in complete DiGeorge anomaly and in *FOXN1* deficiency. Conceptually, the thymus transplant seems to be in principle the more appropriate therapeutic strategy, taking into account that bone marrow transplantation performed in one child with *FOXN1* deficiency, failed to induce a long-term sustained immune reconstitution. In particular, in this patient no reconstitution of the naïve T cell pool was observed (144).

Thymus transplantation has been first used in children affected with complete DiGeorge anomaly, with excellent clinical and immunologic results (141). In order to achieve immune reconstitution, cultured post-natal allogeneic thymus tissue slices were transplanted into the quadriceps muscles of the athymic host (145). The migration of host bone marrow stem cells to the donor graft allow them to develop into naive T cells, which then emigrate out of the engrafted thymic tissue into the peripheral blood. Thymopoiesis is observed in biopsies of the transplanted thymus within 2 months of transplantation (140) and naive T cells are detected in the peripheral blood approximately 3–5 months after transplantation (146, 147). Taking advantage from this previous experience, a few years ago an allogeneic thymus transplantation has been used for the first time in two unrelated infants with Nude/SCID phenotype due to a deficiency of the transcription factor FOXN1 (111). The clinical phenotype of the two subjects was characterized by the absence of naïve T cells, total alopecia, nail dystrophy, and severe infections, as disseminated *Bacillus* Calmette–Guérin in subject 1 and severe respiratory infections in subject 2. Molecular analysis, performed to confirm the clinical suspect of the Nude/SCID phenotype, revealed the presence of a homozygous R255X mutation in the *FOXN1* gene in subject 1, the same of that previously described (107), and a homozygous R320W novel missense mutation in the subject 2. Moreover, subject 1 showed, like a small percentage of complete DiGeorge patients, referred as atypical complete DiGeorge, circulating oligoclonal T cells of non-maternal origins, which were predominantly double-negative T cells, and a T cell proliferative response to PHA within the normal range. Because of that, before thymus transplantation subject 1 have required immunosuppression regimen to prevent graft rejection. Differently, immunosuppression was not used for the subject 2, who had, like typical complete DiGeorge patients, very few T cells (141, 146).

Results obtained with thymus transplantation were encouraging in both FOXN1-deficient subjects, and led to a full T and B cell reconstitution and functional rescue. Indeed, both subjects developed naïve T cells, diverse TCR repertoires and an *in vitro* proliferative T cell responses against different antigens. Eventually they reached normal serum Ig levels with generation of protective antibody specific titers. Of note, HLA matching for class I and II did not seem to interfere with T cell counts after thymus transplantation, being subject 2 transplanted without any HLA matches. However, $CD8^+$ T cell number, although apparently functional, was disproportionally low compared to $CD4^+$ T cells (111). A poor CD8 recovery has also been described in complete DiGeorge patients, who underwent HLA-mismatched thymic transplantation (141, 148). Possible explanations are that the phenomenon is related to the HLA mismatch between host hematopoietic precursors and allograft thymic epithelia or to alterations in the thymic graft due to transplantation procedures.

Functionality of the thymic allograft has been assessed for the first time through signal joint (sj) and DβJβ T cell receptor rearrangement excision circle (TREC) analyses (109). The sj/βTREC represents a ratio between early and late products of TCR rearrangements, which directly correlate with thymic output and provide an indirect measurement of thymocyte division-rate (149–151). The sj/βTREC ratio quantification, conducted in subject 1 with R255X mutation, was very low during the peri-transplant period and comparable to those observed in healthy children at 2.5 years post-transplant. Of note, 4 years post-transplantation a decrease of sj/βTREC ratio associated with a reduction in sjTREC levels and in the number of naïve cells were found, suggesting the decline in thymic allograft output (109). This decline might be due to the reduced longevity of the thymus allograft or to peripheral homeostasis of the T cell pool maintenance following its replenishment. Overall, the thymus transplantation seems to be a promising curative strategy for subjects with athymia due to *FOXN1* deficiency or complete DiGeorge syndrome in the perspective of long-term clinical benefit.

CONCLUSION

The integrity of the thymic epithelial architecture allows the growth, the differentiation, and TCR repertoire selection of immature T cells, thus originating fully mature and functional T cells. Of note, the failure to generate or to maintain the proper 3D thymic architecture leads to severe immunodeficiency or autoimmunity. The unique function of the thymus in the establishment/maintenance of the T cell pool is related not only to the peculiar 3D structure, but also to the specialized functions of the thymic stroma. Indeed, lympho-stromal interactions within the multicellular thymic microenvironment play a crucial role in the regulation of the T cell development. Moreover, these interactions are based on a bilateral crosstalk between stromal cells and traveling thymocytes, which, in turn, are able to provide important signals for the TECs differentiation.

Thymus organogenesis and T cell development are sophisticated biological processes, which require the activation of a wide panel of genes. There is evidence that the master regulator of the thymus development is the *Foxn1* gene, since it is required at

multiple intermediate stages of the TE-lineage specification either in the fetal and adult thymus, through the direct or indirect regulation of genes involved in the thymus development and function. These genes include *Pax1*, *Hoxa3*, *CCL25*, *Dll4*, *p63*.

Studies on the animal and human model of the Nude/SCID phenotype have provided an enormous contribution in identifying the crucial role of *Foxn1* to drive the thymus development, even though many issues regarding the transcriptional regulation of the TECs specification and homeostasis still remain to be solved. The development *in vitro* of cellular models of TEC lineage differentiation, by using the technology of nuclear reprograming, will be certainly useful to better characterize the discrete stages of the TECs differentiation and the molecular mechanism involved in the process.

Eventually, the *in vitro* re-build of a thymic environment capable to reproduce tissue features of primary lymphoid organs (139, 152) could be a promising and valuable tool for the treatment of congenital athymia, including *FOXN1* deficiency, along with the thymus transplantation, which is emerged as a potential treatment for these disorders.

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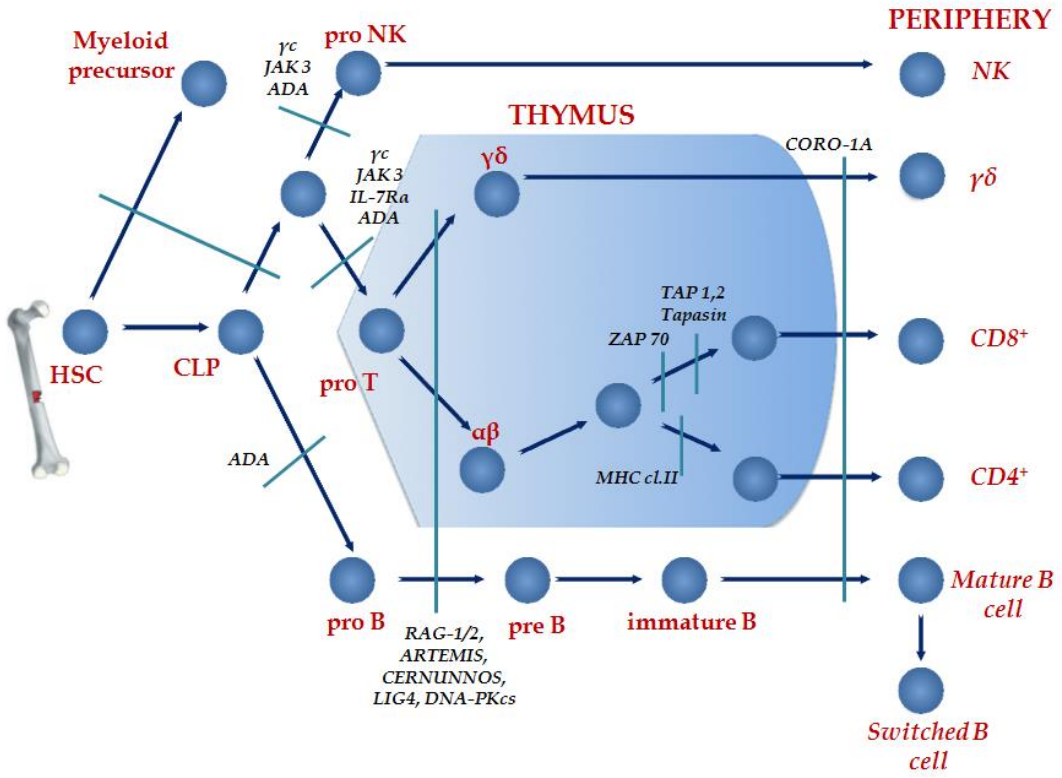
CHAPTER 4

"Rare genetic syndrome involving immune system"

4.1 Severe combined immunodeficiencies – an update

Severe combined immunodeficiencies (SCIDs) includes a number of different inherited disorders characterized by a complete absence or severe dysfunction of the T and B cells, leading to a combined immunodeficiency, which involves both cellular and humoral adaptive immunity. The incidence calculated on the basis of the newborn screening programs in US is 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 (4). SCIDs are classified according to the presence or absence of T, B and NK cells. This classification is representative of the stage where the blockage occurs during the differentiation process and is, therefore, very useful to direct the molecular studies toward a certain genetic alteration (Figure 10).

Figure 10: T, B and NK cells ontogeny in SCIDs. Hematopoietic stem cells (HSC) originate into the bone marrow, which is the only normal site of hematopoiesis in the adult. These cells have self-renewal capacity and can give rise to all the blood cell types from the myeloid and lymphoid lineages. All lymphocytes originate from a common lymphoid progenitor (CLP) that can only give rise to T, B and NK cells. B and NK cells complete their development in the bone marrow, while T cells migrate to and mature into the thymus. Sites of action of different genes involved in lymphocytes ontogeny are indicated. Alterations in single genes affect one or more cell lineage development. Blue lines represent the site where the blockage occurs and the populations affected.



Even though children with SCID appear healthy at birth, early in life, as the maternal transferred antibodies decline, they suffer from severe bacterial, viral or fungal infections. Interstitial lung disease, chronic diarrhea and failure to thrive are the hallmarks of these syndromes at the onset. Skin rashes may be found in some patients as a consequence of transplacental engraftment of maternal T cells during pregnancy or activation of autologous T cells against skin components as a sign of a wide autoreaction (204). According to the ESID diagnostic criteria SCID should be diagnosed in patients who exhibit an absence or a severe reduction of T cells (CD3+ < 300/μL), absence or severe reduction of the proliferative response to phytohemagglutinin (<10% of the lower limit), or a maternal lymphocyte engraftment (17). CID should be diagnosed in 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.

SCIDs are often fatal when the diagnosis is made too late (205). HSCT represents the only curative treatment for most patients (17). To date, gene therapy offers a cure for two specific forms of SCID (206). The conventional classification based on the specific immunologic phenotype may be very useful in directing molecular studies toward a certain genetic alteration. However, the identification of many new causative gene alterations with peculiar clinical and immunological phenotypes and the identification of hypomorphic mutations in some SCIDs genes, and the identification of extra-hematopoietic alterations, which leads to complex phenotypes can make the diagnostic process very complex by standard methods. Taking this into account, the traditional international classification of SCIDs may no longer be optimal for both clinical and research purposes (38, 207).

Recently, T cell receptor excision circles (TREC)–based newborn screening has been introduced in several countries. Patients identified through newborn screening programs, similar to children identified because of a positive familial history, can receive an earlier 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 (208, 209).

In this review, published on *Annals of New York Academy of Sciences,* different forms of SCID and CID, were discussed in detail, 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.

Conclusive remarks

SCIDs are a heterogeneous group of syndromes caused by alterations of distinct genes implicated in the maturation and/or function of T, B, and/or NK cells. The advent of next generation sequencing lead to the identification of new gene alterations responsible of different forms of SCID and CID. 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. This is further complicated by the evidence that different mutations in the same gene can lead to different clinical phenotypes, that may even be inherited with different mechanisms.

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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 $T^+B^+NK^-$ SCID, immune-dysregulation and autoimmunity	Hodgkin like features, invagination and HLH	Not clear; maternal GVHD	55,56
JAK3		Selective $CD4^+$ T lymphopenia	Hypomorphic mutation associated with somatic chimerism	51
RAG	Severe hypogammaglobulinemia, 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 (γc), Janus kinase 3 (JAK3), or the IL-7 receptor α chain (IL-7Rα) are prototypic cytokine-associated disorders, accounting for 67–74% of all cases of SCIDs.[36,37]

Mutations of γc gene cause X-linked SCID (X-SCID), one of the most common forms of SCID, accounting for 50% of all cases. The γc 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 γc 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 γc 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 γc 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 (CD3ε), delta (CD3δ), and zeta (CD3ζ), have been reported in patients with severe forms of SCID, while alterations of the CD3γ 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 γc 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 γ/δ 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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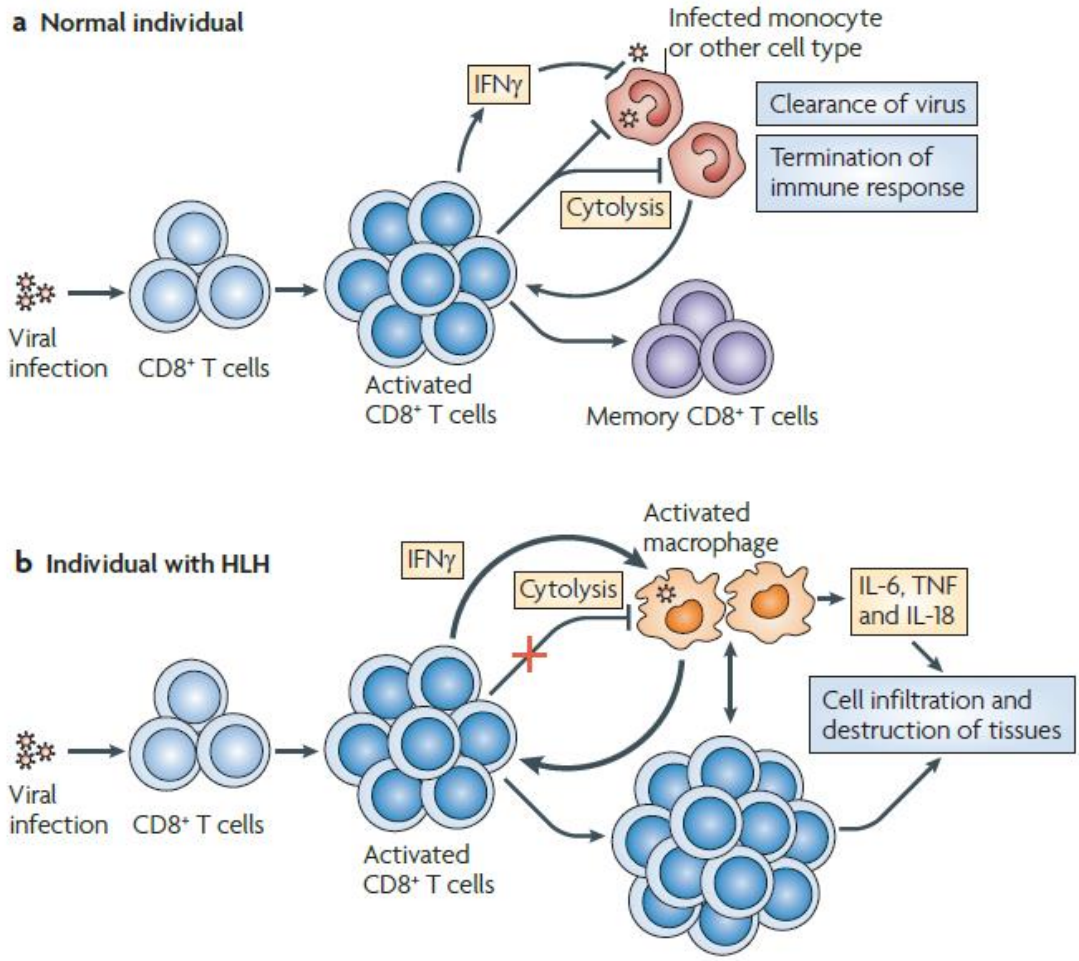
4.2 Phenotypic characterization and outcome of paediatric patients affected with haemophagocytic syndrome of unknown genetic cause

Haemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory condition, potentially fatal, characterized by prolonged and unexplained fever, unresponsive to conventional treatment, hepatosplenomegaly, cytopenia, hypertriglyceridemia and hypofibrinogenemi (210). Histological examination of involved organs typically reveals infiltration of lymphocytes and histiocytes with haemophagocytosis (211, 212). Alterations in six genes, Perforin 1 (PRF1), UNC13D, Syntaxin 11 (STX11), Syntaxin-binding protein 2 (STXBP2), RAB27A and SH2 domain containing 1A (SH2D1A), have been, associated with the familial forms the syndrome. Unrespectively of the etiology, alterations in the mechanisms involved in intracellular trafficking and lysosomal exocytosis, which impair cytotoxic function of T and natural killer (NK) cells, are responsible for the disease pathogenesis. This functional inappropriateness to achieve pathogens clearance leads to a persistent activation and proliferation of cytotoxic T lymphocytes (CTLs) and NK cells (213-215), which, in turn, produce large amount of cytokines and activate histiocytes (macrophages and dendritic cells). The process eventually results in histiocytes tissue infiltration culminating in haemophagocytosis. This syndrome is generally triggered by an infection, notably by a human herpes family virus.

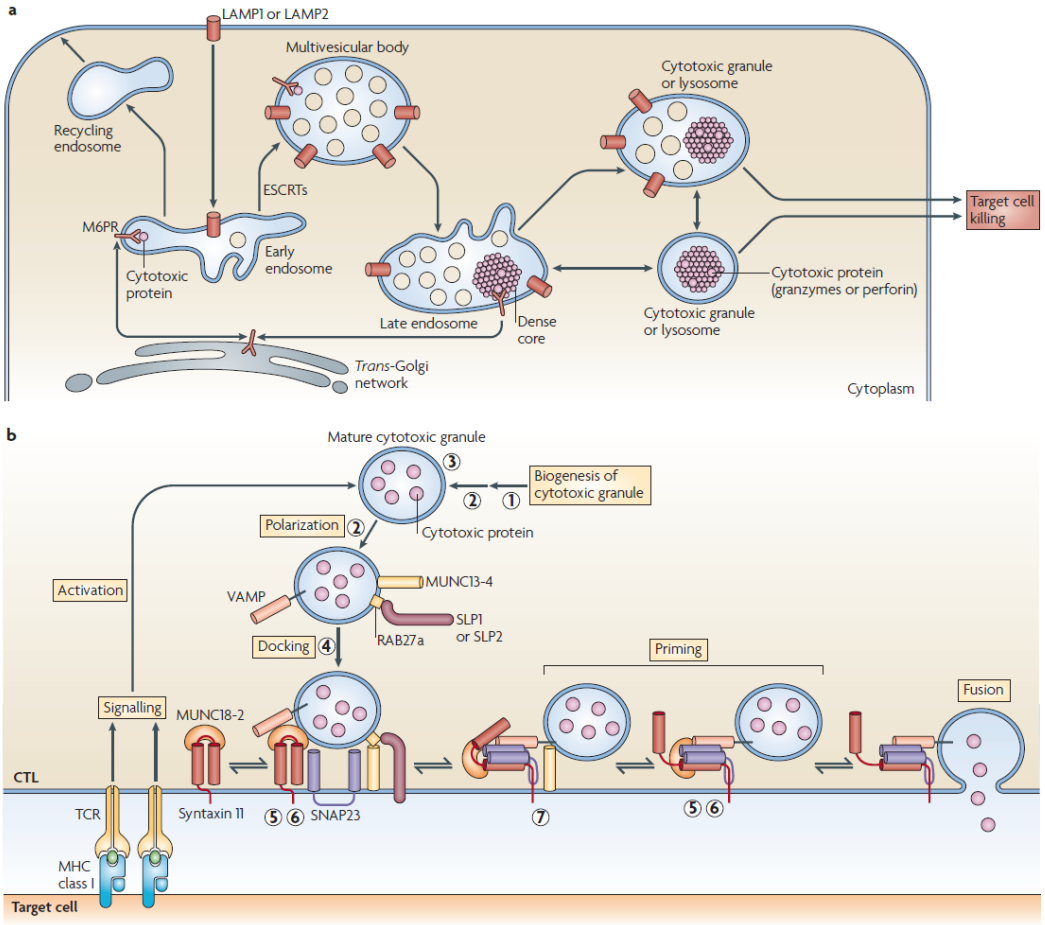
Figure 11: Antigen-specific CD8+ T cell response to viral infection in normal individuals and in patients with haemophagocytic lymphohistiocytosis (216)



Cytotoxic T Lymphocytes and NK cells have a similar mechanism for target cell killing, which occurs through polarized release of the contents of cytotoxic granules towards the target cell. Naive CD8+ T cells require 5–8 days after antigen recognition in secondary lymphoid tissues to differentiate into CTLs, proliferate and migrate to affected tissues. During this differentiation process, granzymes and perforin are synthesized and stored in

developing cytotoxic granules. Lytic granules are secretory lysosomes specialized for the secretion of the cytotoxic effector molecules that function as conventional lysosomes (217-219). Molecules responsible for hemophagocytosis impairs the mechanisms of targeted cell killing, which occurs through different steps including tethering, docking, priming, fusion (Figure 12).

Figure 12: A model depicting the biogenesis and exocytosis of cytotoxic granules (216)



LYST, responsible for Chediack-Higashi syndrome (CHS), is involved in sorting of lysosomal proteins to late endosomes and in regulating fusion or fission of lysosomes.

AP3B1, responsible for type 2 Hermanski Pudlak syndrome (HPS2), encodes AP-3, which is required for the shuttling of cargo proteins from the *trans*-golgi network to endosome- or lysosome-related organelles (220). RAB27a, responsible for type 2 Griscelli syndrome (GS2), is important regulators of vesicle trafficking and compartmentalization. Patients affected with CHS, HPS2 and GS2 also show hypopigmentation, resulting from aberrant formation of melanosomes. *PRF1*, responsible for type 2 FLH, encodes perforin, which is required for the delivery of granzymes into the cytoplasm of the target cell. MuNC13-4, responsile for FHL3, mediates the tethering of cytotoxic granules to the plasma membrane. Fusion of the cytotoxic granules to the plasma membrane is mediated by SNARE proteins, including Syntaxin 11 and MuNC18-2, responsible for FHL4 and FHL5, respectively.

In the majority of cases a genetic defect cannot be identified. In a few of these cases without a known genetic alteration, various infectious, malignant and autoimmune triggers have been suggested as implicated in the pathogenesis (221).

A clinical and laboratory characterization of HLH has been provided mainly for genetic forms. In this study, published *Letter to the Editor* on *British Journal of Hematology,* we characterized the clinical and laboratory features and the outcome of pediatric patients diagnosed as HLH of unknown genetic cause, in the attempt to define clinical and laboratory hallmarks to help distinguish these patients from those with a known genetic cause. Compared to what is known in the familial cases, median age at onset was higher in HLH than in FHLH (222). A predominance of affected males was observed in our cohort with a male-to-female ratio of 1.6:1. The difference in male-to-female ratio seems to rely predominantly on the underlying disorder. The clinical course of paediatric HLH with

unknown genetic alteration is in general more favorable then FHLH, as suggested by later onset, lower prevalence of neurological involvement and better outcome.

Cancer Institute ALL 91-01 Consortium used a higher cumulative dose of Peg- Asparaginase (30 000 iu/m^2) (Kearney *et al*, 2009). Asparaginase dose intensity is however, not the only factor likely to be important in the pathogenesis of pancreatitis. Regimen B patients had a three-fold higher incidence of pancreatitis compared to Regimen A despite having a similar Asparaginase exposure. This suggests that anthracycline induction and the additional cylophosphamide/cytarabine exposure in the Berlin-Frankfürt-Münster consolidation in Regimen B may be risk factors for the development of pancreatitis.

In our series, Asparaginase withdrawal due to pancreatitis did not affect EFS and OS, though the overall numbers of cases with pancreatitis was small. However, in the current UKALL 2011 trial, we continue to recommend that Asparaginase be withheld following pancreatitis. Our analysis demonstrates that increasing treatment intensity and Asparaginase exposure are the biggest risk factors for developing pancreatitis.

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Authorship contributions

RW, SD, PI and JS collected data and helped write the manuscript. RW performed the statistical analyses. AJ, NG and SS supervised the writing of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary information.

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Phenotypic characterization and outcome of paediatric patients affected with haemophagocytic syndrome of unknown genetic cause

Haemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory condition caused by a highly stimulated, but ineffective, immune response (Trottestam *et al*, 2011). To date, alterations in six genes have been identified in the

Table I. Demographic and clinical findings of 21 patients with haemophagocytic lymphohistiocytosis.

Case	Age at onset (years)	Gender	Comorbidity	Infectious trigger
1	8.7	F	Bibasic aminoaciduria; jaundice; alveolar proteinosis	–
2	0.4	M	–	–
3	1.9	F	–	–
4	6.2	M	–	+ (Staphylococcus, EBV)
5	15.0	M	Hodgkin lymphoma	–
6	1.6	F	Langerhan hystiocytosis	+ (EBV)
7	5.0	F	Visceral leishmaniosis	–
8	14.2	M	Gitelman syndrome	–
9	5.0	M	Chronic granulomatous disease	+ (Staphylococcus)
10	2.1	M	–	–
11	4.6	M	Visceral leishmaniosis	–
12	0.2	M	–	–
13	0.6	M	Reumathoid artrhitis	–
14	1.2	F	Muscolar glicogenosis	–
15	0.7	M	–	–
16	0.8	M	–	+ (unidentified)
17	0.8	F	Acute myeloid leukaemia	+ (CMV)
18	2.4	M	Acute myeloid leukaemia	–
19	1.4	F	–	+ (Parvovirus)
20	2.3	M	–	–
21	2.5	F	–	–

F, female; M, male; EBV, Epstein-Barr virus; CMV, cytomegalovirus.

familial forms. However, in the majority of cases the genetic defect cannot be identified. Studies in humans indicate that alterations in the mechanisms involved in intracellular trafficking and lysosomal exocytosis, which impair cytotoxic function of T and natural killer (NK) cells, may be implicated in the pathogenesis. The inappropriateness in pathogens clearance leads to persistent activation and proliferation of cytotoxic T lymphocytes (CTLs) and NK cells (Crozat *et al*, 2007), which, in turn, produce large amount of cytokines and activate histiocytes.

This study aimed to retrospectively characterise the clinical and laboratory features and outcome of 21 paediatric patients diagnosed as HLH of unknown genetic cause, in the attempt to define clinical and laboratory hallmarks to help distinguish these patients from those with a known genetic cause.

Patients alive, with no disease activity for >1 year off-therapy, were considered as non-familial HLH (FHLH) (Trottestam *et al*, 2011). We also included three patients who underwent haematopoietic stem cell transplantation (HSCT) after disease reactivation, as all the known causes of FHLH were excluded through molecular or functional analysis. Detailed materials and methods are provided in the supporting information section.

Mean age at disease onset was 3.7 years (range: 2 months–15 years), higher than in FHLH, where onset is within the first year of life in 70–80% of cases (Trottestam *et al*, 2011). Six patients (28%) were diagnosed during the first year of life.

Differently from FHLH, which is inherited as a recessive trait, thus implying the same probability to be affected for males and females, a predominance of affected males was observed in our cohort (male-to-female ratio: 1.6:1), in keeping with previous reports of adult patients with secondary HLH, mainly affected with haematological malignancies (Shabbir *et al*, 2011). In contrast, female sex was reported to be more frequent in patients with Epstein–Barr virus (EBV) triggered HLH (Trottestam *et al*, 2011), thus suggesting a role for the underlying disorder rather than to the pathogenetic mechanism for the gender prevalence. Comorbidities were found in 52% of the patients (visceral leishmaniosis, 9.5%; haematological malignancies, 18.9%; other genetic-metabolic diseases, 18.9%; rheumatoid arthritis, 4.7%) (Table 1). The association between HLH and several underlying disorders, including autoimmune diseases, malignancies, primary or acquired immunodeficiencies, metabolic disorders and infections (Kaito *et al*, 1997) is a well-documented feature. In six patients, a viral or bacterial infection preceded the onset of a HLH overt clinical manifestation. The most common infectious triggering agents were herpes viruses, mainly EBV and cytomegalovirus (CMV), although bacteria, protozoa and fungi may also be involved. In particular, Leishmania infection, may mimic or trigger HLH with a still poorly understood mechanism (Rajagopala *et al*, 2008).

All patients had fever at onset, while hepatomegaly and splenomegaly were observed in 91% and 71% of patients, respectively; 86% of the patients had anaemia at diagnosis, with a mean haemoglobin of 77 ± 10.5 g/l, and 90% had

Table II. Incidence of pre- and post-treatment outcome predictors.

	Pre-treatment outcome predictors					Post-treatment outcome predictors						
	Jaundice	Bilirubin >51.3 μmol/l	Ferritin >2000 μg/l	AST >400 U/l	LDH >600 U/l	Fever	Platelet count <40 × 10^9/l	Ferritin >2000 μg/l	Hb <80 g/l	AST >60 U/l	Fibrinogen <1.5 g/l	Treatment
1	+	+	+	+	+	–	–	+	+	+	+	HSCT
2	–	–	+	–	+	–	–	–	–	–	–	Ciclosporin, steroid
3	+	+	+	+	+	–	–	–	–	+	+	Ciclosporin, steroid, etoposide
4	–	–	+	–	+	–	+	–	–	–	–	Steroid
5	+	+	+	+	+	–	+	NA	–	–	NA	HSCT
6	–	–	NA	–	+	–	+	NA	–	–	–	Ciclosporin, steroid, etoposide
7	–	–	+	–	+	–	–	–	–	–	–	Amphotericin-B, steroid
8	–	–	–	–	+	–	–	–	–	–	+	Ciclosporin, steroid
9	–	NA	NA	–	NA	–	–	NA	–	–	NA	Steroid, etoposide
10	–	+	NA	–	+	–	–	–	–	–	+	Ciclosporin, steroid, etoposide
11	–	–	–	–	+	–	–	–	–	+	+	Amphotericin-B, steroid
12	–	NA	+	+	+	–	–	–	–	–	–	–
13	–	–	+	–	+	–	+	+	–	–	–	Ciclosporin, steroid, etoposide
14	–	–	NA	–	–	–	NA	NA	NA	NA	NA	Ciclosporin, steroid, etoposide
15	–	–	+	+	+	–	–	–	–	–	–	Steroid
16	–	–	–	–	–	–	+	NA	–	+	–	HSCT
17	–	–	–	–	+	–	–	–	–	–	–	–
18	–	–	+	+	+	–	–	–	–	+	–	Steroid, etoposide
19	–	–	+	–	+	–	–	NA	–	–	NA	Ciclosporin, steroid, etoposide
20	–	–	+	–	+	–	–	–	–	–	–	Steroid
21	–	NA	+	–	+	–	–	–	–	+	+	Ciclosporin, steroid, etoposide

AST, aspartate transaminase; LDH, lactate dehydrogenase; Hb, haemoglobin; NA, not available, HSCT, haematopoietic stem cell transplantation.

thrombocytopenia (mean platelet count, 57.5 ± 37.75 × 10^9/l). Only 14% of patients had a tri-lineage cytopenia, the most common feature of FHLH (Kaito *et al*, 1997; Trottestam *et al*, 2011). Neurological involvement, a prominent feature in about 50% of patients with FHLH (Trottestam *et al*, 2011), was found only in the 9% of our patients as seizures, somnolence or rigour nucalis. Of note, liquor examination was always negative. Bone marrow examination revealed haemophagocytosis in 71% of the cases. Patients were treated according to HLH-04 protocol. Seven patients (33%) required a 3-drug regimen, four patients (20%) achieved a full remission with a 2-drug treatment (steroid and ciclosporin or steroid and etoposide) and three patients (14%) only with steroids. The two patients with leishmaniosis achieved remission after amphotericin-B and steroid. Two patients had a spontaneous remission. Three patients underwent a successful HSCT (Table II). In the two patients with the neurological involvement at onset, a full recovery was observed in one patient, while seizures persisted in the other one.

A recent case series, analysing risk factors for early death in children with HLH, documented that jaundice, hyperbilirubinaemia, hyperferritinaemia, elevated aspartate transaminase (AST) and lactate dehydrogenase levels at onset, and fever, thrombocytopenia, hyperferritinaemia, anaemia and elevated AST and fibrinogen levels after 2 weeks of therapy were associated with worse outcome (Trottestam *et al*, 2012). In our cohort (Table II), 18/21 patients had three or less negative outcome predictors before the start of the therapy. Among the patients with more than three negative outcome predictors, two underwent a successful HSCT, while the third achieved a full remission after 3-drug regimen. Regarding the post-treatment outcome predictors evaluation, the only patient who had more than three negative outcome predictors pre- and post-treatment, required HSCT.

Perforin (*PRF1*) expression on peripheral blood NK cells ($CD3^-CD56^+$) was impaired in four of 17 tested patients and in three of them the molecular analysis revealed a heterozygous *PRF1* A91V mutation, whose relevance in the disease pathogenesis remains controversial (Busiello *et al*, 2004, 2006). Recent studies suggest that *PRF1* A91V genotype is present in the normal population, thus being insufficient *per se* to cause FHLH, although it may represent a genetic susceptibility factor for immune dysregulation (Zhang *et al*, 2007). One of these patients also carried a heterozygous *UNC13D* R967E mutation.

In eight patients and 13 controls, the degranulation of NK cells, through CD107a expression, and IFNγ production after incubation with K562 line cells was evaluated. The percentage of $CD107a^+CD56^+CD3^-$ cells and $IFN\gamma^+CD56^+CD3^-$ lymphocytes <10th percentile of normal values were considered reduced. CD107a expression in $CD56^+CD3^-$ population revealed a lower percentage of $CD107a^+$ cells in the patients as compared with the controls (49.98 ± 7.43% vs. 84.60 ± 2.07%, respectively; $P < 0.0001$). Moreover, patients showed a significantly lower percentage of $IFN\gamma^+$ cells than controls (20.89 ± 3.57% vs. 62.61 ± 4.20%, respectively; $P < 0.0001$). Of note, all patients had a low percentage of $IFN\gamma^+$ NK cells; only one of them exhibited a normal CD107a expression. Studies on mouse models indicate that defects in the production of IFNγ or cytotoxicity may render mice more susceptible to CMV infection (Lee *et al*, 2007). This would suggest that abnormal intracellular trafficking, along with a reduction in the IFNγ production, could be implicated in the pathogenesis of HLH under condition of challenge by viral infections or other types of environmental stress.

In conclusion, the clinical course of paediatric HLH with unknown genetic alteration is more favorable then FHLH, as suggested by later onset, lower prevalence of neurological involvement and better outcome. Given that HLH is a potentially life-threatening condition shared by distinct unrelated clinical entities, the definition of the underlying common pathogenetic mechanism is mandatory to better define the management of such patients and open new therapeutic options.

Authorship Contributions

GG, CV, RN, EC, and VG selected the cases, analysed and interpreted the data, and wrote the manuscript. GR, VR and AP performed and reviewed the degranulation and IFNγ production assays and critically revised the manuscript. VP critically revised the manuscript. CD selected the cases and critically revised the manuscript. CP selected the cases, analysed and interpreted the data, reviewed the degranulation and IFNγ production assays and wrote and critically revised the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials and methods.

Fig S1. Functional analysis. Panel (A) There was a significant lower percentage of $CD107a^+$cells in the patients as compared with the controls (49.98 ± 7.43% vs. 84.60 ± 2.07%, respectively; $P < 0.0001$). Panel (B) A significant lower percentage of γIFN^+ cells was observed in the patient cohort, than in controls (20.89 ± 3.57% vs 62.61 ± 4.20%, respectively; $P < 0.0001$).

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More on normal prothrombin times in the presence of therapeutic levels of rivaroxaban – early experience from King's College Hospital

We read with interest the letter by van Veen *et al* (2013) describing their experience on the performance of the Innovin® and Thromborel S® prothrombin time assays in a 74-year-old patient with renal impairment who was commenced on rivaroxaban for the treatment of deep vein thrombosis. It was interesting to observe that with both assays, the prothrombin time (PT) was not prolonged, despite therapeutic concentrations of rivaroxaban, and we thank van Veen and colleagues for highlighting this important observation to the wider clinical community. To complement their report, we would like to share our experience of how a different PT assay to those utilized by van Veen *et al* (2013) performed in a group of patients attending King's College Hospital who were prescribed rivaroxaban.

During the past 6 months, we have been prescribing novel oral anticoagulants (NOAC) for selected patients, if they fit the local South London Cardiac and Stroke network criteria for NOAC use (http://www.slcsn.nhs.uk/noacs.html). For these patients, when clinically indicated (e.g. extremes of body weight, renal dysfunction, or presence of interacting drugs), we measured the activity of the rivaroxaban, in order to satisfy ourselves that the desired response was being achieved. Our laboratory uses the STA-Neoplastine® (Diagnostica Stago, Asnières-sur-Seine, France) CI Plus PT assay for detection of the presence of the drug (rivaroxaban), and the STA-liquid anti-Xa assay® (Diagnostica Stago, Asnières-sur-Seine, France), with appropriate rivaroxaban calibrators and controls for quantification of the actual rivaroxaban concentration in the patients plasma.

Table I describes the results of the anti-Xa rivaroxaban concentrations, along with the respective PT assay results from 18 patients in whom we measured these activities. In all cases (except Patient 10), the patients had been prescribed

4.3 Intergenerational and intrafamilial phenotypic variability in 22q11.2 Deletion syndrome subjects

Chromosome 22q11.2 deletion syndrome also known as DiGeorge syndrome (DGS) (OMIM#188400), is a rare disorder due to chromosome 22q11.2 deletion. Although most cases of DGS occur as de novo deletions, approximately 5% of cases are inherited in an autosomal dominant pattern (223-227). The incidence has been estimated at 1:4000 live births but recent evidence indicates that the syndrome is more common than previously thought (228, 229). In fact, an increasing number of individuals with mild features has been identified carrying the 22q11.2 deletion. Major clinical features include facial anomalies, conotruncal cardiac defects, palatal anomalies, neonatal hypocalcaemia, mild to moderate immune deficiency related to thymic a/hypoplasia (230-232), developmental and speech delay (233). Ocular, renal and skeletal anomalies may also be found. Psychiatric or autoimmune disorders (234) can be the features leading to the diagnosis in adolescents and adults, and, in particular, among adults, they may be the unique clinical feature (235, 236). However, the syndrome has a very wide spectrum of phenotypic features (224, 237). The identification of subjects with attenuated phenotypes is leading to the understanding that the syndrome is more frequent than previously thought and to focus on novel atypical presentations. A wide clinical variability has also been reported even within the same family (223, 238-240) and a phenotypic discordance has been described among monozygotic twins (241, 242). Genetic modifiers, chance association or environmental interactions have been proposed to explain the intrafamilial variability. Somatic mosaicism or post zygotic second

hit have also been hypothesized as potential mechanisms underlying such phenotypic discordance, even though, to date, no definitive explanation is available.

In this study published on *BMC Medical Genetics,* realized in collaboration with the Centers member of Italian Primary Immunodeficiencies Network (IPINET), we compared the intergenerational variations of the clinical phenotype between patients affected with DGS and their affected parents carrying 22q11.2 deletion.

Conclusive remarks

A more severe clinical phenotype characterized by highest prevalence of life-threatening manifestations in the last generation, suggesting the worsening of the phenotype generation by generation was observed. This evidence highlights the importance for the clinicians to look for milder manifestations in order to suspect the diagnosis of DGS and for the physicians to better define the relationship between clinical phenotype and genetic alteration in DGS.

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Intergenerational and intrafamilial phenotypic variability in 22q11.2 Deletion syndrome subjects

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Abstract

Background: 22q11.2 deletion syndrome (22q11.2DS) is a common microdeletion syndrome, which occurs in approximately 1:4000 births. Familial autosomal dominant recurrence of the syndrome is detected in about 8-28% of the cases. Aim of this study is to evaluate the intergenerational and intrafamilial phenotypic variability in a cohort of familial cases carrying a 22q11.2 deletion.

Methods: Thirty-two 22q11.2DS subjects among 26 families were enrolled.

Results: Second generation subjects showed a significantly higher number of features than their transmitting parents (212 vs 129, $P = 0.0015$). Congenital heart defect, calcium-phosphorus metabolism abnormalities, developmental and speech delay were more represented in the second generation ($P < 0.05$). Ocular disorders were more frequent in the parent group. No significant difference was observed for the other clinical variables. Intrafamilial phenotypic heterogeneity was identified in the pedigrees. In 23/32 families, a higher number of features were found in individuals from the second generation and a more severe phenotype was observed in almost all of them, indicating the worsening of the phenotype over generations. Both genetic and epigenetic mechanisms may be involved in the phenotypic variability.

Conclusions: Second generation subjects showed a more complex phenotype in comparison to those from the first generation. Both ascertainment bias related to patient selection or to the low rate of reproductive fitness of adults with a more severe phenotype, and several not well defined molecular mechanism, could explain intergenerational and intrafamilial phenotypic variability in this syndrome.

Keywords: 22q11.2 deletion syndrome, DiGeorge syndrome, Immunodeficiency, Phenotypic variability

Background

Chromosome 22q11.2 deletion syndrome (22q11.2DS), also known as DiGeorge or velocardiofacial syndrome (OMIM#188400), occurs in approximately 1:4000 live births [1,2]. Major clinical features include facial anomalies, conotruncal cardiac defects, palatal anomalies, neonatal hypocalcaemia, mild to moderate immune deficiency related to thymic a/hypoplasia [3-5], developmental and speech delay [6]. Ocular, renal and skeletal anomalies may also be found. However, the syndrome has a very wide spectrum of phenotypic features [7,8]. Evidence highlighted that subjects carrying the deletion may have only mild phenotypes [9,10]. Psychiatric or autoimmune disorders [11] can be the features leading to the diagnosis in adolescents and adults, and, in particular, among adults, they may be the unique clinical feature [12,13]. The identification of subjects with attenuated phenotypes is leading to the understanding that the syndrome is more frequent than previously thought and to focus on novel atypical presentations. A wide clinical variability has also been reported even within the same family [9,10,14,15] and a phenotypic discordance has been described among monozygotic twins [16,17]. Genetic modifiers, chance association or environmental interactions have been proposed

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to explain the intrafamilial variability. Somatic mosaicism or post zygotic second hit have also been hypothesized as potential mechanisms underlying such phenotypic discordance, even though, to date, no definitive explanation is available.

The deletion results from non allelic homologous recombination, occurring during meiosis and mediated by low-copy repeats (LCR) on chromosome 22 [18-20]. Most patients have a deletion of the same 3 Mb region on 22q11.2, including about 30 genes, whereas in 8% of the cases a smaller deletion of 1.5 Mb, which contains 24 genes, is found. So far, no correlation between the severity of the phenotype and the different size of the deletion has been documented [21]. Both deletions include the *TBX1* gene, a member of the T-box family genes. Mice, haploinsufficient for *TBX1,* share several features with humans carrying the homologous deletion, and, in particular, structural cardiac anomalies [22]. Interestingly, both gain or loss of function mutations in *TBX1* have been reported in human subjects exhibiting a DiGeorge-like phenotype [23].

In most cases, the deletion is a sporadic event, while in 8-28% of the cases the syndrome is inherited in an autosomal dominant fashion [8,10,24-26].

Several studies have analyzed the phenotypic variability of the syndrome, but an extensive and conclusive intergenerational and intrafamilial comparison has not yet been reported.

The aim of this study is to perform an intergenerational and intrafamilial comparison of the clinical phenotype in a cohort of patients affected with inherited chromosome 22q11.2DS.

Methods

Patients

Thirty-two subjects (18 females) affected with familial 22q11.2DS from 26 families, were enrolled into the study. The study and data collection, approved by the local Ethics Committee for Biomedical activities "Carlo Romano", have been performed upon informed consent and in compliance with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/index.html). Within the group, 17 subjects were from the Italian Network for Primary Immunodeficiencies (IPINET) Registry, followed at 8 Italian Centers, and 15 were referred to two Genetic Units. In 4 families, 2 affected siblings were diagnosed and in one further family, 3 subjects were identified. Mean age +/− SD was 10.4 +/− 7.23 years (range 4 months-31 years). The parent carrying the deletion was the mother in 17 cases (65%) and the father in 9 (35%). Mean age +/− SD of carrier parents was 39.8 +/− 7.9 years (range 21–58 years). We found a preferential maternal transmission, in keeping with the recent observation that female sex represents a significant positive predictor of fitness. All the parents were identified as affected by 22q11.2DS after the birth of a child with the deletion. All patients, but two, were Caucasian. Demographical features are reported in Additional file 1: Table S1. Clinical data of the second generation subjects were obtained, upon informed consent, through the IPINET Registry or from the referring Units. Data on parents carrying the deletion were collected at each Center. Each subject underwent a clinical and laboratory evaluation protocol (IPINET protocol for 22q11.2DS) available at the site http://www.aieop.org. The protocol included cardiologist examination, echocardiography and abdominal ultrasound, which were performed in order to exclude subclinical cardiac or abdominal defects in asymptomatic subjects. To exclude thyroid and calcium-phosphorus abnormalities, the serum levels of calcium, phosphorus, parathyroid hormone, TSH and FT4 were evaluated. History of speech therapy or speech pathologist interventions were recorded for the evaluation of language disorders. The Wechsler Intelligence Scale for Children (WISC) and the Wechsler Adult Intelligence Scale (WAIS) were used for the assessment of cognitive function in subjects of the second generation and in their parents, respectively. Each Center reported the presence/absence of intellectual disability which was defined as an IQ under 70. Neuropsychiatric evaluation was performed by skilled clinicians using the Schedule For Affective Disorders and Schizophrenia for School-aged Children, Present and Lifetime (K-SADS-PL). The second generation subjects, older than 18 years, and their parents were interviewed with the Structured Clinical Interview for Axis I DSM IV Disorders (SCID).

Among the clinical features, birth defects, facial anomalies, gastrointestinal disorders, infections and autoimmune manifestations were recorded. History of neonatal hypocalcaemia was also considered. In 6 families, data were also obtained from the non-deleted parent, to exclude potential interfering factors not related to the 22q11.2 deletion.

Intrafamilial phenotypic variability was assessed through the evaluation of the clinical phenotype in each parent–child couple. Since in 5 families more than 1 subject with the inherited deletion was detected, the phenotype was analyzed in a total number of 32 parent–child pairs from the 26 families.

Cytogenetic analysis

The diagnosis of 22q11.2DS was performed by fluorescence *in situ* hybridization (FISH) analysis and/or multiplex ligation-dependent probe amplification (MLPA) using probes for 22q11 region in all affected patients. In one case, the diagnosis was obtained through a CGH array with whole-genome oligonucleotide microarray Agilent Technologies (Santa Clara, CA) according to the manufacturer protocol.

Statistical analysis

Statistical analysis was performed using the Student"s *t* test or the Fisher exact Test. Values of $P < 0.05$ were considered statistically significant. The calculations were performed using InStat software.

Results

Intergenerational clinical phenotypic comparison

Seventeen clinical variables were evaluated in subjects of the second generation and their parents (Table 1), for a total number of 544 and 442 variables in the first and second group, respectively. Overall, affected subjects of the second generation showed a significantly higher number of features than their parents (212 vs 129, $P = 0.0015$). In particular, congenital heart defect (CHD) (62.5 vs 7%, $P < 0.0001$), developmental delay (71.8 vs 42.3%, $P = 0.032$), speech delay (75 vs 46.1%, $P = 0.031$) and calcium-phosphorus abnormalities (37.5 vs 3.8%, $P = 0.0033$) were more represented in the second generation. Conversely, ocular disorders were more frequent in the parents than in their affected children (3.1 vs 23%, $P = 0.037$). Psychiatric (12.5 vs 34.6%), autoimmune (12.5 vs 19.2%) and dental disorders (25 vs 38.4%) tended to be more frequent in the older generation, even though the differences did not reach a statistical significance. No statistically significant difference was observed for the other phenotypic features. About 15% of the clinical features were diagnosed during the study.

Table 1 Clinical characteristics of second generation subjects and parents carrying the 22q11.2 deletion

	Second generation	Parents	
Total number of subjects	**32**	**26**	
	N (%)	**N (%)**	**p**
Facial anomalies	29 (90.6)	24 (92.3)	1
Congenital heart defect	20 (62.5)	2 (7)	**<0.0001**
Ca-P abnormalities	12 (37.5)	1 (3.8)	**0.0033**
Palatal anomalies	18 (56.2)	13 (50)	0.79
ENT anomalies	4 (12.5)	2 (7)	0.68
Renal disorders	7 (21.8)	2 (7)	0.16
Ocular disorders	1 (3.1)	6 (23)	**0.037**
Neurological disorders	3 (9.3)	1 (3.8)	0.62
Dental anomalies	8 (25)	10 (38.4)	0.39
Skeletal anomalies	15 (46.8)	10 (38.4)	0.6
Gastrointestinal disorders	8 (25)	2 (7)	0.16
Psychiatric disorders	4 (12.5)	9 (34.6)	0.06
Language delay	24 (75)	12 (46.1)	**0.031**
Developmental delay	23 (71.8)	11 (42.3)	**0.032**
Learning difficulty	23 (71.8)	16 (61.5)	0.57
Autoimmunity	4 (12.5)	5 (19.2)	0.71
Infections	9 (28.1)	3 (11.5)	0.19

Only severe infections (sepsis, pneumoniae), requiring hospitalization, or history of recurrent infections were considered. Bold indicate *P* values considered statistically significant.

We next compared the anatomic type of CHD between the 2 groups. We found that in the 20% of the subjects of the second generation with CHD, each individual patient had more than 1 abnormality, whereas in the parent group none of them had a more severe defect. A cyanotic CHD was found only in the 35% of the group with the cardiac defect. In particular, among these patients, we found that 7/20 subjects of the second generation exhibited a tetralogy of Fallot (TOF) and only 1/20 truncus arteriosus (TA). Among those with non cyanotic CHD, a patent ductus arteriosus (PDA) was observed in 4/20, atrial septal defects (ASD) in 4/20, interrupted aortic arch (IAA) in 4/20, ventricular septal defects (VSD) in 3/20, pulmonary valve stenosis (PVS) in 1/20 and other in 2/20. In the parent group, the anomalies found were a PDA in one case and a double aortic arch (DAA) in the other one.

With regard to calcium-phosphorus metabolism abnormalities, 9 subjects in the second generation presented with neonatal hypocalcemia, and in 2 of them a hypoparathyroidism was later diagnosed. Overall, at any age, a total of 5/32 (15.6%) of them received a full diagnosis of hypoparathyroidism. Only 1 subject of the parent group was affected with asymptomatic hypoparathyroidism.

Although the prevalence of the palatal defects was similar in the 2 groups (56 and 50%, respectively), we next compared the type of the defect and made a comparison. Within the group of patients of the second generation, 1 subject had cleft palate and bifid uvula, 16 velopharyngeal insufficiency, 4 hypernasal speech and 4 high arched palate. In the parent group, 11 had velopharyngeal insufficiency, 3 hypernasal speech, 1 high arched palate. Only in two parents, a cleft palate was observed. Thus, no remarkable difference was found for these variables.

Psychiatric disorders were more frequent in the parent group, even though the difference did not reach statistical significance ($P = 0.06$). We found that in the parent group, anxiety was observed in 5/26, mood disorders in 3/26, attention-deficit/hyperactivity disorders in 3/26, behavioral anomalies in 3/26, schizophrenia in 2/26, psychotic disorders not otherwise specified (NOS) in 2/26 and phobia in 1/26. Within the second generation group, 2 of them had behavioral abnormalities represented by trend to social isolation and rejection, impairment in social and daily living skills and low self-esteem, 1 also showed adaptive abnormalities, and the other one an attention deficit disorder. Schizophrenia was observed in 1 subject of the second generation, who, however, was 31 years-old.

Ocular defects were the only anomaly frequently observed in the parental generation, consisting in refractive defects (3/6), strabismus (2/6), retinal vessel abnormalities

(1/6), cataract (1/6) and xerophthalmia (1/6). The only child with ocular defect had retinal vessels abnormalities.

Intrafamilial clinical phenotypic comparison

With regard to the intrafamilial phenotypic variability, in 23 out of the 32 child/parent couples a higher number of features was found in the second generation, although in 6 couples the number of features was higher in the parents' generation and in the remaining 3 couples no difference was found (Figure 1). None of the couples with higher number of features in the parents' generation had a CHD or hypoparathyroidism.

We then performed an intrafamilial evaluation of the clinical severity of the phenotype with regard to the prominent clinical features, whose prevalence was statistically different between the 2 groups, namely CHD, anomalies of the calcium-phosphorus metabolism, developmental and/or speech delay (Table 2).

In particular, we observed that CHD was present in 18 couples only in the second generation and, in one case, only in the first generation. In the couple in which both members were affected, the parent exhibited a PDA, whereas his child was affected with IAA, thus confirming the lack of phenotypic correspondence. Calcium-phosphorus abnormalities were found in 11 subjects of the second generation, and the only parent with asymptomatic hypoparathyroidism had a child who presented with hypocalcaemia. In 15 couples speech delay was exclusively observed in the second generation whereas in 5 couples only in the first generation. In the remaining 9 couples both members were affected. In 16 couples, developmental delay was exclusively found in the second generation, in 4 cases only in the first generation and in 8 couples both subjects were affected.

To rule out the interference of factors not related to the 22q11.2 deletion, in 6 families, data were collected also from the non-deleted parent. Of note, none of them had a CHD, while 2 children in this subgroup were affected. One subject reported learning and behavioral problems, but not intellectual disabilities, while in another case a borderline IQ of 68 was determined.

Furthermore, in the families with more than one affected child, a milder phenotype was observed in parents than in children, even though the 5 subjects of the second generation, who were diagnosed first, had a higher number of the major clinical features compared to their 6 siblings (16 core features vs 11).

Discussion and conclusions

We have compared the clinical phenotype of a cohort of 32 subjects affected with inherited 22q11.2DS and their transmitting parents.

In this study, we found a higher number of clinical features and a more severe phenotype in the second generation, which exhibited a higher number of more severe conditions. CHD, abnormalities of the calcium-phosphorus metabolism, developmental and/or speech delay were more represented in children than in parents. It should be considered that, in the past decades, severe CHD was associated with a high neonatal and infant mortality. This evidence was thought to have an appreciable impact on reducing the reproductive fitness of 22q11.2DS patients with CHD. More recently, however, with the improvement of cardiac surgery strategies, it is suggested

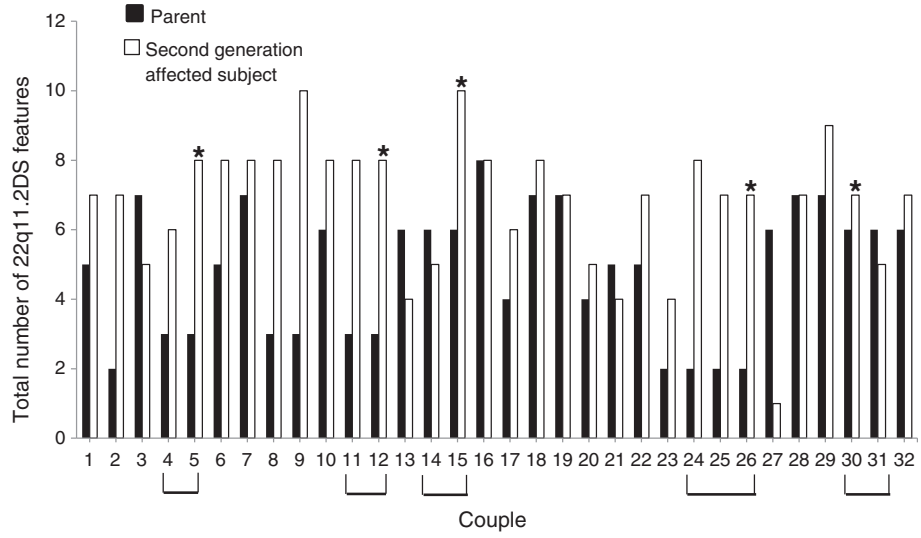

Figure 1 Intrafamilial phenotypic comparison. A higher number of features were observed in the second generation in 23 couples. In 6 couples the number of features was higher in the parents' generation and in the remaining 3 couples no difference was found. The brackets indicate the families with 2 or more second generation affected subjects. * indicates the proband within these families.

Table 2 Intrafamilial comparison of the clinical severity of the phenotype

Couple	Congenital heart defect		Ca-P abnormalities		Speech delay		Developmental delay	
	Affected subject	Parent	Affected subject	Parent	Affected subject	Parent	Affected subject	Parent
1	yes	no	yes	yes	no	yes	yes	no
2	yes	no	yes	no	yes	no	no	no
3	yes	no	no	no	no	yes	yes	no
°4	no	no	yes	no	no	yes	yes	no
°5[δ]	yes	no	yes	no	yes	yes	yes	no
6	yes	no	no	no	yes	yes	yes	yes
7	yes	no	yes	no	yes	no	yes	no
8	no	no	no	no	yes	no	yes	no
9	no	no	no	no	yes	no	yes	no
10	no	yes	yes	yes	yes	yes	no	no
°11	yes	no	yes	no	no	no	yes	no
°12[δ]	yes	no	no	no	yes	no	yes	no
13	no	no	no	no	yes	no	no	yes
°14	no	no	no	no	yes	no	yes	yes
°15[δ]	yes	no	no	no	yes	no	no	yes
16	no	no	no	no	yes	no	yes	no
17	yes	no	no	no	yes	yes	yes	yes
18	yes	no	no	no	yes	yes	yes	yes
19	yes	no	no	no	yes	yes	no	yes
20	yes	yes	no	yes	yes	yes	yes	no
21	yes	no	yes	no	yes	yes	yes	yes
22	yes	no	yes	no	yes	yes	yes	yes
23	yes	no	yes	no	yes	no	yes	yes
°24	no	no	no	no	yes	no	yes	no
°25	no	no	no	no	yes	no	yes	no
°26[δ]	yes	no	yes	no	yes	no	yes	no
27	yes	no	yes	no	no	yes	no	yes
28	no	no	no	no	yes	yes	yes	yes
29	no	no	no	no	yes	no	yes	no
°30[δ]	yes	no	no	no	yes	no	yes	no
°31	no	no	no	no	no	no	no	no
32	yes	no	no	no	no	no	no	no

°Couple of siblings; [δ]First individual to seek medical attention for genetic evaluation.

that a stronger negative selective pressure against the transmission of 22q11.2 deletion is primarily due to the severity of the neuropsychiatric phenotype and intellectual disabilities [27].

The only anomalies more frequent in the parental generation were ocular abnomalities. Since most of them, in particular refractive defects and cataract usually develop during older age, we could not exclude a bias related to the different ages of the subjects in the two groups.

When an intrafamilial comparison of the phenotypic complexity was performed, a higher prevalence of clinical features were found in the second generation. When the comparison concerned the prominent clinical features, whose prevalence was statistically different between the 2 groups, namely CHD, calcium-phosphorus metabolism anomalies, developmental delay and speech delay, we observed that in almost all parent/child couples these major features were more frequent in the second generation. In previous studies [9,10,13-15,24], an intrafamilial

variability has already been reported, even though the comparison of intergenerational clinical features has not been performed. An ascertainment bias could partially explain this finding, since the first subject diagnosed within a family is likely to be more severely affected. Moreover, our observation may also be explained by a bias related to the low rate of reproductive fitness of adults with a more severe phenotype.

As to developmental delay, the presence of environmental intellectual disabilities may obviously *per se* influence the mental development of the offspring, because of psychosocial deprivation. However, it should be emphasized that in our cohort the majority of the subjects with developmental delay have a parent without intellectual disability. Moreover, in the 6 families in which also the non-deleted parent was studied, the lack of a clear correlation between environment and the child' development was noted. As expected, psychiatric disorders were more represented in the first generation, however, the difference was not significant thus suggesting the need of an accurate psychiatric management since childhood.

Several genes, such as *TBX1* [28], *HIRA, UFD1L* [29] and *CRKL* [30], within the 22q11 region have been considered to be implicated in the pathogenesis of the syndrome. The 22q11.2 phenotype is a developmental field defect, and a DiGeorge-like phenotype may also occur in the absence of the deletion [31], as in diabetic [32] and retinoic acid embryopathy, fetal alcohol syndrome, CHARGE [33] and Fraser syndromes, as well as other chromosomal anomalies, such as 10p13, 17p13, 4q34.1q35.2 [34-36], indicating that several molecules in a common genetic pathway or in functionally related pathways may be involved in 22q11.2DS clinical manifestations.

Several hypotheses have so far been proposed to explain intergenerational and intrafamilial phenotypic variability in genetic syndromes. Deletions of different sizes and location and the extension of an unstable mutation at the 22q11.2 locus could explain the clinical variability [37].

Evidence indicates that *TBX1* gene is sensitive to altered dosage [38], thus leading to the hypothesis that additional alterations of the other allele may explain the clinical variability. In humans, this does not seem to be the case, in that so far DNA variations in *TBX1* locus on the remaining allele were not found in 22q11.2DS patients exhibiting a variable cardiovascular expression and palatal defects. Thus, it is likely that gene modifiers not related to chromosome 22 may be implicated [39,40].

The increased risk of cardiac defects observed in unaffected relatives of 22q11.2DS subjects with CHD, suggested a potential role for genes outside the DiGeorge critical region [41,42]. However, studies aimed at identifying genetic factors outside of the 22q11,2 region, such as *VEGFA* [43] or folate-related genes [44] failed to reveal any association. It should be noted that in the subgroup of families in whom also the non-deleted parent was studied, no CHD was noted suggesting the absence of interfering genetic factors not related to the 22q11.2 deletion in this context.

A copy-number variation may explain a reduced penetrance of some disease-causing mutations [45]. A genetic compensatory effect has also been documented in families of 22q11.2DS subjects, whose clinically normal parent carried 22q11.2 deletion compensated by an insertion of the 22q11.2 critical region inside the other copy of the chromosome [46]. Finally, a mosaic status in the carrier parent, even though rare, could be the explanation of the variable and more benign phenotype. At the moment, we cannot yet exclude the presence of a concomitant duplication or copy number variations in the former generation, since further studies with interphase FISH or array-CGH are required to rule out this hypothesis.

During development, gene expression is accurately orchestrated in time and space in a program that involves enzymes controlling nucleosome remodeling, histone modification and DNA methylation [47]. A demonstration that an epigenetic alteration could result in a DiGeorge syndrome phenocopy has been recently documented in mutant mice lacking the MOZ histone acetyltransferase [48]. Thus, failure of these fine tuned mechanisms could result in an interference of the phenotypic expression.

In our study we also observed that some clinical features were more represented in the previous generation, although the difference did not reach the statistical significance for the limited number of subjects studied. In keeping with recent findings, we observed a higher incidence of psychiatric disorders in the older generation.

The identification of adults with a milder phenotype deserves careful attention since a later onset illness associated with 22q11.2DS has been reported, highlighting the possibility that along with psychiatric disorders, also treatable conditions such as symptomatic or asymptomatic hypocalcaemia, thrombocytopenia and hypothyroidism may occur [49]. Early recognition of these features [50] could provide the benefit of an early treatment [51-53].

Advantages and limitations of the study

This is the largest cohort of subjects affected with familial 22q11.2DS. A detailed characterization of the clinical features of such subjects, and an intergenerational/intrafamilial clinical comparison has been performed. The observation that within the families, the patients who were first diagnosed had a higher number of core features as compared to their siblings or parents would suggest an ascertainment bias, even though the clinical phenotype of the parents was milder compared to their children. Another possible explanation could be the co-inheritance of a further genetic defect from the non-affected parent. This seems unlikely since this co-inheritance should have

occurred in all cases exhibiting the worsening of the phenotype. As for the CHD, it should be noted that none of the non-affected parents had a CHD, thus excluding, at least for this feature, this hypothesis.

Additional file

Additional file 1: Table S1. Demographic characteristics of the 22q11.2DS subjects.

Abbreviations

22q11DS: 22q11.2 Deletion syndrome; LCR: Low-copy repeats; IPINET: Italian network for primary immunodeficiencies; WISC: Wechsler intelligence scale for children; WAIS: Wechsler adult Intelligence Scale; FISH: Fluorescence in situ hybridization; MLPA: Multiplex ligation-dependent probe amplification; CGH: Comparative genoma hybridization; CHD: Congenital heart defect; TOF: Tetralogy of Fallot; TA: Truncus Arteriosus; PDA: Patent Ductus Arteriosus; ASD: Atrial septal defects; IAA: Interrupted Aortic Arch; VSD: Ventricular septal defects; PVS: Pulmonary valve stenosis; DAA: Double aortic arch.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CE conceptualized and designed the study, enrolled patients and collected data, drafted the initial manuscript. GG, VG enrolled patients, collected data and coordinated data collection from each group, drafted the initial manuscript. PP, CA, FB, MPC, RC, SM, BM, CM, VM, AP, GS,CC, PR enrolled patients, collected the data, and revised the manuscript. MCD enrolled patients, collected data and critically revised the manuscript. CP conceptualized and designed the study, enrolled patients, collected data and drafted the initial manuscript. MCD and CP equally contributed to the paper. All the authors approved the final version of the manuscript.

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4.4 Gastrointestinal involvement in patients affected with 22q11.2 deletion syndrome.

Gastrointestinal (GI) alterations, including feeding disorders (237, 243) and congenital abnormalities (224), are often reported in patients with Del22 and other athymic disorders (188, 231). The commonest disorders are: gastroesophageal reflux disease (GERD), esophagitis and chronic constipation. Feeding disorders, mostly due to dysmotility in the pharyngoesophageal area, are characterized by difficulties in coordinating the suction/deglutition/breathing reflex and result in slow feeding and episodes of regurgitation (237, 243). The GI congenital abnormalities include: esophageal atresia, jejunal atresia, umbilical hernia, diaphragmatic herniation, intestinal malrotation, congenital megacolon, anorectal malformations (atresia, anterior displacement) and dental anomalies, such as delayed formation and eruption of permanent teeth and enamel hypoplasia (224). However, a systematic and well detailed clinical and laboratory characterization of the GI alterations in Del22 syndrome is missing. In this monocentric study published on *Scandinavian Journal of Gastroenterology* we defined the GI involvement in a cohort of patients affected with Del22.

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ORIGINAL ARTICLE

Gastrointestinal involvement in patients affected with 22q11.2 deletion syndrome

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Abstract

Objective. Enteropathy is a very common feature in patients with primary immunodeficiencies. In patients with Del22 gastrointestinal (GI) alterations, including feeding disorders and congenital abnormalities have been often reported, mostly in the first year of life. ***Material and methods.*** Aim of this monocentric study is to better define the GI involvement in a cohort of 26 patients affected with Del22 syndrome. Anamnestic information was retrospectively collected for each patient. Weight and height parameters at the time of the screening were recorded. Plasma levels of hemoglobin, iron, ferritin, albumin, total protein, calcium, phosphorus, transaminase levels, antigliadin (AGA) IgA and IgG, and antitissue transglutaminase (anti-TGase) titers were measured. ***Results.*** A GI involvement was identified in the 58% of patients. The prominent problems were abdominal pain, vomiting, gastroesophageal reflux and chronic constipation. Weight deficiency, short stature and failure to thrive were reported in 54, 42, and 30% of the patients, respectively. The evidence of sideropenic anemia, in keeping with hypoproteinemia, impaired acid steatocrit or cellobiose/mannitol test suggested an abnormal intestinal permeability. In this cohort, a high prevalence of AGA IgA and IgG positivity was observed. Celiac disease (CD) was suspected in three patients, and in one of them confirmed by histology. In this patient, a long-lasting gluten-free diet failed to restore the intestinal architecture. ***Conclusions.*** In conclusion, GI involvement is a very common feature in Del22 patients. A better characterization of GI involvement would be very useful to improve the management of these patients.

Key Words: *Celiac disease, Del22 syndrome, malabsorption*

Introduction

Chromosome 22q11 deletion (Del22) is the most common chromosomal deletion syndrome with an estimated incidence of 1:4,000 live births [1]. The most common clinical features of the syndrome are cardiac malformations, speech delay, facial dismorphisms, and immunodeficiency [2]. Other manifestations include neuropsychiatric, gastrointestinal (GI) and otolaryngological disorders.

GI alterations, including feeding disorders [3,4] and congenital abnormalities [5], are often reported in patients with Del22 and other athymic disorders [6,7]. The commonest disorders are gastroesophageal reflux disease (GERD), esophagitis, and chronic constipation. Feeding disorders, mostly due to dysmotility in the pharyngoesophageal area, are characterized by difficulties in coordinating the suction/deglutition/breathing reflex and result in slow feeding and episodes of regurgitation [3,4]. The GI congenital abnormalities include esophageal atresia, jejunal atresia, umbilical hernia, diaphragmatic herniation, intestinal malrotation, congenital megacolon, anorectal malformations (atresia, anterior displacement), and dental anomalies, such as delayed formation and eruption of permanent teeth and enamel hypoplasia [5]. However, a systematic and well-detailed clinical and laboratory characterization of the GI alterations in Del22 syndrome is missing. Aim of this monocentric study is to better define the

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GI involvement in a cohort of patients affected with Del22.

Methods

Twenty-six patients affected by Del22 syndrome in follow-up at our Department were enrolled into the study. The clinical diagnosis of Del22 syndrome was confirmed by fluorescent *in situ* hybridization (FISH). For each patient, anamnestic information, paying a special attention to chronic diarrhea, nausea and vomiting, abdominal pain, GERD, esophagitis, chronic constipation and feeding difficulties were retrospectively collected. Weight and height parameters at the time of the screening were recorded and plotted on standard percentile growth charts and the closest percentile for age was recorded. As previously reported [8], the finding of weight below the 3rd percentile was correlated with the presence of congenital heart defect, cleft palate, and feeding difficulties. The finding of short stature (height below the 3rd percentile) was correlated with the presence of congenital heart defect and cleft palate. Statistical analysis was performed through a two tailed Fishers exact test using the statistical software GraphPad.

Plasma hemoglobin, iron, ferritin, albumin, total protein, calcium, phosphorus, and transaminase levels were measured. Stool samples were collected for the measurement of fecal calprotectin, as a marker of bowel inflammation, and for the acid steatocrit estimation, as a marker of small-intestine lipid malabsorption. The cellobiose/mannitol test, performed by standard procedure, was used to investigate intestinal permeability. Results were expressed as the ratio (cellobiose/mannitol) of the 5 h urinary recovery of the two probe molecules. The test was considered impaired when cellobiose/mannitol ratio was >0.023. To rule out GI infections, parasitological test and stool cultures were conducted.

All patients were receiving a gluten-containing diet. Enzyme-linked immunosorbent assay (ELISA) was used to measure antigliadin (AGA) IgA and IgG, and antitissue transglutaminase (anti-TGase) titers. IgA and IgG AGA titers were considered negative when <45 U/mL, and positive when >55 U/mL. IgA and IgG anti-TGase titers were considered negative when <1 U/mL, borderline when 1–6.9 U/mL and positive when >7 U/mL. The indirect immunofluorescence technique on commercially available fixed sections of monkey small intestine was used for endomysial antibody (EMA) determination. IgA levels were evaluated in each patient in order to exclude a selective IgA deficiency. According to the ESPGHAN criteria, esophagogastroduodenoscopy (EGDS) was proposed when anti-TGase antibodies were positive, but with a title <10 times the upper normal value; in children younger than 2 years, EGDS was proposed when AGA titers were positive even though in the absence of anti-TGase antibodies. EGDS was performed upon written informed parental consent.

Results

Patients affected with Del22 syndrome had a mean age of 7.6 years (age range 0.6–20.7 years). Male to female ratio was 1.4:1. Four cases carried an inherited deletion, while in 22 cases the deletion was *de novo*. Table I shows the main clinical and demographic features of the patients enrolled into the study. In 15/26 patients (58%) clinical records revealed the presence of signs or symptoms of a GI involvement. Mean age at the onset of GI symptoms was 1.7 years (age range 0–7 years). Abdominal pain was reported in 9/15 patients, vomiting in 8/15, chronic constipation in 7/15, GERD in 6/15, feeding disorders in 4/15, failure to thrive in 4/15, epigastric pain in 3/15, and diarrhea in 4/15 (Table II). Two patients had congenital abnormalities, such as gastrectasia or anorectal malformation. All patients showed two or more symptoms.

Weight deficiency (<3rd centile) was found in 14/26 (54%) patients, short stature (<3rd centile) in 11/26 (42%) and failure to thrive (weight/height ratio <3rd centile) in 8/26 (30%). Congenital heart defect was present in 13 of the 14 (93%) patients with weight below the 3rd percentile and in 9 of the 12 (75%) patients with the weight within the normal range (p 0.30). Cleft palate was present in 1 of 14 (7%) patients with weight below the 3rd percentile and in 2 of 12 (16%) patients with weight within the normal range (p 0.58). Feeding difficulties were referred in 1 of 14 (7%) patients with weight below the 3rd percentile and in 1 of 12 (8%) of those with weight above the 3rd percentile (p 1.00). Congenital heart defect was present in 11 of 11 (100%) patients with height below the 3rd percentile and in 11 of 15 (73%) patients with height above the 3rd percentile (p 0.11).

Hematological and biochemical tests revealed hypoproteinemia in 5/26 patients. In 3/5 cases, hypoproteinemia was associated with GI symptoms, including diarrhea in two cases and feeding difficulties in one case. Sideropenic anemia was observed in 14/26 patients, while one additional patient had hyposideremia and hypoferritinemia without anemia. In 5/14 patients, sideropenic anemia was not associated with an overt GI manifestation.

IgA and IgG AGA titers were positive in the 41 and 47% of the patients, respectively. Anti-TGase IgA titers were positive in 4.5% of the patients, borderline in 32% of the patients, and negative in

Table I. Clinical and demographic features of patients.

Pz	Age (years)	Sex	Transmission	Clinical features
1	6.2	M	AD	cardiac anomalies, mental retardation
2	14.0	M	*de novo*	hypoparathyroidism, recurrent candidiasis
3	16.7	M	AD	cardiac anomalies, mental retardation
4	10.2	M	AD	cardiac anomalies, mental retardation
5	6.0	M	*de novo*	cardiac anomalies, mental retardation, neonatal tetanus, URTI
6	12.5	M	*de novo*	cardiac anomalies, mental retardation
7	11.5	F	*de novo*	cardiac anomalies, mental retardation, hypoparathyroidism, URTI, subdural hygroma
8	7.5	F	*de novo*	cardiac anomalies, mental retardation, URTI, febrile seizures, pulmonary atelectasis
9	12.5	F	*de novo*	cardiac anomalies
10	9.0	F	*de novo*	cardiac anomalies
11	20.7	F	*de novo*	cardiac anomalies, hypoparathyroidism, psoriasic arthritis, hepatitis C
12	4.5	F	*de novo*	cardiac anomalies, mental retardation
13	1.2	F	*de novo*	cardiac anomalies, gastrectasia, recurrent oral candidiasis
14	7.0	M	*de novo*	cardiac anomalies, mental retardation, autoimmune thyroiditis
15	3.5	F	AD	cardiac anomalies
16	4.5	F	*de novo*	cardiac anomalies, recurrent oral candidiasis, URTI
17	3.5	M	*de novo*	cardiac anomalies, mental retardation, neonatal tetanus, seazures
18	3.2	M	*de novo*	mental retardation, palatal defect
19	7.7	M	*de novo*	mental retardation
20	10.5	M	*de novo*	cardiac anomalies, mental retardation, thrombocytopenia, myopia, cryptorchidism, inguinal hernia, velopharyngeal synechia
21	4.5	M	*de novo*	cardiac anomalies, febrile seizures, recurrent bronchospasm, inguinoscrotal hernia
22	10.5	F	*de novo*	mental retardation, palatal defect, hypocalcemia, seizures
23	6.5	M	*de novo*	cardiac anomalies, mental retardation, hexodactily, hypoacusia, cryptorchidism, URTI, febrile seizures
24	0.7	F	*de novo*	cardiac anomalies, IgA deficiency
25	2.0	M	*de novo*	cardiac anomalies, palatal defect, URTI
26	0.6	M	*de novo*	cardiac anomalies, inguinal hernia, skeletal malformations

Abbreviations: AD = autosomal dominant; URTI = upper respiratory tract infection.

63.5%. Anti-TGase IgG titers were positive in 8% of the patients and negative in 92%. None of the patients had EMA. Celiac disease (CD) was suspected on the basis of serological tests in one patient, who showed persistently elevated anti-TGase IgA, and in two patients younger than 24 months, who had positive IgA and IgG AGA titers. The three patients with a positive serology had GI signs suggestive of CD (chronic diarrhea, abdominal pain, and failure to thrive), and only one patient had sideropenic anemia. As mentioned earlier, EGDS was performed when indicated, but one patient who refused the procedure. In two patients, aged 10 and 14 months, respectively, the intestinal histological examination revealed a villous atrophy consistent with a diagnosis of CD in one patient and a normal architecture in the second one. In the patient with villous atrophy, a moderate-to-severe villous atrophy persisted after a 7-year gluten-free diet. Thereafter, a liberalized diet did not impair the growth (weight to height ratio at the 50th percentile).

Acid steatocrit estimation was impaired in 6 of the 18 patients evaluated. Fecal calprotectin was impaired in 8 of the 22 patients evaluated. The cellobiose/mannitol test, performed in 14/26 patients, was impaired in four patients. These assays were impaired contemporarily in three patients (P3, P8, and P21), who had GI clinical signs. In the overall group with altered intestinal permeability, five patients had GI clinical signs. By contrast, six of the eight patients, who had increased calprotectin levels, had GI signs.

In 12/26 patients, at least two further autoantibodies, not directly associated with the intestine, were detected (Table III).

Discussion

Patients with primary immunodeficiencies are prone to develop enteropathy with different pathogenetic mechanisms. In patients with Del22, mostly in the first year of life, GI alterations, including feeding disorders [3,4] and congenital abnormalities [5] have been already reported [8].

In this study, a GI involvement was identified in the 58% of patients affected with Del22. The most frequent clinical problems were abdominal pain, vomiting, GERD, and chronic constipation. In our cohort, weight deficiency, short stature, and failure to thrive were reported in 54, 42, and in 30% of the patients, respectively. In keeping with what previously

Table II. Clinical and laboratory GI evaluation in Del22 patients.

Pz	GI signs and symptoms	Age at onset	P/A <3rd pc	TP (g/dl)	Alb (g/dl)	Iron (μg/dl)	FT (ng/ml)	Hb (g/dl)	Ca (mg/dl)	P (mg/dl)	AST (UI/l)	ALT (UI/l)
1	–	–	+	6.6	4.5	15.0	44.0	11.0	9.3	4.5	21.0	17.0
2	–	–	–	7.8	4.9	51.0	23.0	11.4	8.7	5.1	18.0	16.0
3	+ (diarrhea, vomiting, failure to thrive, abdominal pain)	1.3	+	5.4	3.2	14.0	19.0	12.1	8.4	4.3	22.0	19.0
4	+ (CMPI, chronic constipation, abdominal pain)	1.8	–	7.0	4.4	54.0	44.0	12.6	8.6	4.5	24.0	16.0
5	+ (diarrhea, GERD)	0.2	–	5.6	3.7	15.0	7.0	10.1	9.2	4.1	52.0	21.0
6	+ (nausea, vomiting)	1.7	+	7.0	3.6	15.0	8.0	10.1	9.3	4.3	75.0	59.0
7	–	–	–	5.9	3.7	13.0	5.0	9.6	7.2	5.0	21.0	17.0
8	+ (epigastric pain, abdominal pain, chronic constipation)	7.0	–	6.6	4.2	14.0	5.0	9.2	9.0	5.5	23.0	20.0
9	+ (chronic constipation, dyspepsia, epigastric pain, vomiting, abdominal pain)	1.2	–	7.0	4.7	77.0	13.0	11.7	9.0	3.0	26.0	21.0
10	+ (epigastric pain, abdominal pain)	2.0	–	7.6	4.4	14.0	9.0	10.3	9.3	4.9	24.0	17.0
11	–	–	–	7.7	4.5	15.0	7.0	10.8	7.5	3.6	25.0	19.0
12	+ (nausea, vomiting, diarrhea, failure to thrive, abdominal pain)	1.2	+	7.4	4.6	15.0	10.0	9.3	10.3	7.6	21.0	17.0
13	+ (feeding difficulties, gastrectasia)	0.5	+	5.8	2.4	59.0	13.0	11.4	9.7	6.2	23.0	19.0
14	+ (failure to thrive, vomiting, GERD, chronic constipation, anorectal malformation)	3.0	–	7.0	4.3	14.0	7.0	10.0	9.1	4.4	22.0	17.0
15	+ (chronic constipation, vomiting, GERD, feeding difficulties)	0.5	+	6.3	4.4	14.0	7.0	9.9	9.2	5.3	31.0	22.0
16	+ (vomiting, failure to thrive, diarrhea, abdominal pain, GERD)	2.2	–	6.9	4.2	32.0	15.0	11.1	9.0	5.1	22.0	18.0
17	–	–	–	6.5	4.3	60.0	17.0	12.0	9.6	5.4	26.0	22.0
18	–	–	–	6.7	4.4	12.0	11.0	9.9	9.5	5.2	29.0	15.0
19	–	–	+	7.3	4.6	100.0	26.0	12.0	9.0	5.2	24.0	11.0
20	–	–	–	7.4	4.8	108.0	35.0	13.0	9.0	4.1	22.0	16.0
21	+ (abdominal pain, vomiting, chronic constipation, GERD)	3.0	–	6.9	4.4	15.0	11.0	10.7	9.5	5.3	31.0	12.0
22	–	–	–	7.5	4.2	60.0	20.0	11.6	8.7	4.2	23.0	17.0
23	–	–	+	6.9	4.5	15.0	18.0	10.8	9.5	4.4	24.0	21.0
24	–	–	–	5.4	4.4	15.0	5.0	9.5	10.1	5.6	55.0	23.0
25	+ (GERD, feeding difficulties)	0.1	–	6.8	4.1	58.0	53.0	11.5	9.6	5.5	32.0	19.0
26	+ (chronic constipation, abdominal pain, feeding difficulties)	0.1	–	6.1	4.5	63.0	7.0	9.3	10.5	6.8	31.0	15.0

Abbreviations: Alb = albumin; Ca = calcium; CMPI = cow's milk protein intolerance; FT = ferritin; GERD = gastroesophageal reflux disease; Hb = hemoglobin; P = phosphorus; Pc = percentile; TP = total protein.

Table III. Serological markers of autoimmunity.

PZ	AGA IgA (U/ml)	AGA IgG (U/ml)	Anti-TGasi IgA (U/ml)	Anti-TGasi IgG (U/ml)	EMA	ANA	Anti-dsDNA	ENA	Anti-TG	Anti-TPO	CIC C1q	CIC C3d	C3	C4
1	–	–	–	+ (13.1)	NA	+	+	+	–	–	+	+	+	+
2	–	NA	–	–	NA	–	–	–	–	–	+	+	+	–
3	+ (1362.0)	+ (1771.0)	± (1.3)	+ (8.0)	–	+	+	–	–	–	+	+	+	+
4	+ (400.0)	+ (400.0)	–	–	–	+	+	+	+	–	+	+	+	+
5	+ (150.0)	+ (125.0)	± (1.7)	–	–	+	+	+	–	–	+	+	+	+
6	–	–	–	–	NA	+	+	NA	–	–	+	+	+	+
7	+ (384.0)	+ (400.0)	–	–	–	+	–	+	–	–	+	+	+	+
8	+ (196.0)	+ (77.0)	± (2.2)	–	–	+	–	–	–	–	+	+	+	+
9	+ (298.0)	+ (400.0)	± (2.8)	–	–	+	+	+	–	–	+	+	+	+
10	+ (200.0)	–	+ (8.0)	–	–	–	–	NA	–	–	+	+	+	–
11	–	NA	–	–	NA	–	–	–	–	–	NA	–	–	–
12	+ (81.0)	+ (400.0)	–	–	–	–	–	–	–	–	+	+	–	+
13	–	NA	–	–	NA	–	–	–	–	–	NA	–	–	–
14	+ (320.0)	+ (97.0)	–	–	–	+	–	NA	+	+	+	+	+	+
15	–	–	–	–	NA	+	–	+	–	–	+	+	–	–
16	–	–	± (2.5)	–	NA	+	+	+	+	–	+	+	+	+
17	–	+ (78.0)	–	–	–	–	–	NA	–	–	+	+	+	+
18	–	–	–	–	NA	+	–	+	–	–	+	+	+	NA
19	–	–	–	–	NA	+	–	+	–	–	+	+	+	–
20	–	–	NA	–	NA	–	–	NA	–	–	–	–	–	NA
21	NA	–	± (1.6)	–	–	NA	–	–	–	–	–	–	+	+
22	–	NA	–	–	NA	–	–	NA	–	NA	+	+	+	+
23	NA	–	± (2.0)	–	NA	+	–	NA	–	–	+	+	+	+
24	NA	NA	NA	NA	NA	NA	NA	NA	–	–	–	NA	NA	NA
25	–	NA	–	–	NA	–	–	–	–	–	+	+	–	–
26	–	–	–	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Abbreviations: AGAs = antigliadin antibodies; ANAs = antinuclear antibodies; anti-dsDNAs = antidouble-stranded DNA antibodies; anti-TGs = antithyroglobulin antibodies; anti-TGasis = antitransglutaminase antibodies; anti-TPOs = antithyroperoxidase antibodies; CICs = circulating immune complexes; EMAs = antiendomisium antibodies; ENAs = extractable nuclear antigen antibodies; + = positive; – = negative; ± = borderline; NA = not available.

reported, the presence of congenital heart defect or cleft palate has no impact on the overall growth patterns. In fact, the correlation between the presence of these risk factors and low weight or short stature in our cohort of patients was not statistically significant. Contrary to what was previously reported, feeding difficulties do not seem to predispose to growth abnormalities. However, it should be noted that in our cohort of patients, only few of them had feeding difficulties, thus making difficult any statistical comparison.

In this study, we identified a high prevalence of AGA IgA and IgG positivity. Studies on Italian students, aged 11–15 years, revealed a prevalence of AGA positivity of 2% [9,10]. An elevated prevalence of AGA positivity has already been reported in different genetic disease [11–13], such as Down syndrome [14,15] and Williams syndrome. In these patients, this finding was initially explained by an impaired intestinal permeability, which is a well-documented feature in patients with congenital immunodeficiencies as a consequence of the increased susceptibility to infections. In our cohort, the presence of sideropenic anemia in 14 patients may suggest an iron malabsorption, in keeping with hypoproteinemia, impaired acid steatocrit or cellobiose/mannitol test observed in six of them. The underlying mechanism to explain abnormal intestinal permeability in these patients is, however, distinct from the mechanism implicated in CD, in that in CD

the altered permeability is associated with permeability genes variations, which was not found in other conditions associated with AGA positivity [15,16]. Since in Del22 syndrome the number of GI infections is usually not higher than controls, other immunologic phenomena may be implicated in the increased prevalence of elevated AGA, possibly involving altered induction and/or maintenance of tolerance [15,17]. Patients affected with immunodeficiency, and, in particular, patients affected with Del22 are particularly prone to develop autoimmune disorders [18]. In our cohort, 7/9 patients with AGA positivity had at least 1 further autoantibody, not directly related to intestinal autoimmunity, even though none of them showed an overt clinical autoimmune disorder.

An increased incidence of CD has already been reported in Del22 patients [19]. The criteria to suspect a CD were present in three patients, but none of the patients had EMA. Moreover, a long-lasting gluten-free diet failed to restore the intestinal architecture, indicating a different pathogenetic mechanism for such histological alterations. Intestinal villous atrophy mimicking CD has been observed in the 31% of common variable immunodeficiency (CVID) patients with GI symptoms or anemia (12). The enteropathy of CVID patients significantly differs from typical forms of CD. In particular, the resistance to gluten-free diet is common, while steroids are effective on GI symptoms and in reducing the mucosal damage. Similar to CVID, the enteropathy associated with Del22 is nowadays poorly characterized, and its possible relationship with the well-defined pathogenetic mechanism of the enteropathy in CD is still poorly investigated.

In conclusion, GI involvement is a very common feature in Del22 patients. A better characterization of GI involvement would be very useful to improve the management of patients with Del22.

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TECHNOLOGIES

§ Patients

Concerning the study on NGS, patients, heterogeneous for ethnic origin, age and sex, were accurately selected according to clinical and immunological criteria, highly suggestive for primary immunological defect. The clinical criteria adopted included one or more of the following: opportunistic infections; granuloma; CMC; intractable diarrhea; bronchiectasis; severe autoimmunity; variably associated to syndromic features and/or familiarity for similar phenotype. Any clinical criteria was considered if associated to one or more of the following quantitative and/or qualitative immunological criteria: abnormal lymphocyte subsets (absolute count < 2 SD of normal values according to ESID criteria); proliferative response to mitogens <10% of the CTR; absent/poor specific antibody response; hypogammaglobulinemia; high IgE levels (>2000 KU/l); absent cytolytic activity; alteration of class switch recombination (CSR) with or without hyper-IgM.

Concerning the study on CMCD, patients carrying *STAT1* GOF mutations were prospectively included in the study from January 2011 to January 2015. They were from 30 different countries. Physicians caring for the patients completed a detailed questionnaire, after the eligible patient had agreed to participate and was enrolled in the study. Relatives of patients with *STAT1* GOF mutations who displayed CMCD but could not be genotyped (deceased, lost to follow up) were considered to carry the respective *STAT1* GOF mutations and were included in the study. Asymptomatic relatives of included patients were genotyped when possible. Data were collected for the patients, from their birth until January 2015 or

their death. Data were centralized at the coordinating center at Necker-Enfants Malades University Hospital. Patients with CMCD for whom no *STAT1* mutation was identified during the study were excluded.

§ Cells and cell cultures

Peripheral Blood Mononuclear cells (PBMC) were obtained from patients and healthy donors by Ficoll-Hypaque (Biochrom) density gradient centrifugation. Fibroblasts were isolated by mincing of dermal skin fragments and were grown in Dulbecco's modified Eagle's medium (Invitrogen) and 10% FBS (Gibco), supplemented with 100 U/ml Penicillin and 100 μg/ml Streptomycin (Invitrogen). The cultures were incubated at 37°C in the atmosphere supplemented with 5% CO2, with the cell culture media changed daily. Fibroblasts were passaged fewer than 5 times before use in experiments.

To obtain polyclonal NK cell lines, NK lymphocytes were isolated from PBMCs using negative selection (NK cell isolation Kit, Miltenyi), then cultured on irradiated feeder cells in the presence of 100 U/mL recombinant human IL-2 (Proleukin; Chiron) and 1.5 ng/mL phytohemagglutinin (Gibco). CD4+ cells were separated from the patient and a healthy control by positive selection using human CD4 microbeads.

U3C cells, a STAT1-deficient fibrosarcoma cell line, were grown in DMEM (EuroClone) and 10% FBS (Gibco), supplemented with 2mM L-Glutammine, 1000U/m Penicilline and Streptomycin (EuroClone).

§ DNA extraction and sequence capture array design

DNA was isolated from peripheral blood lymphocytes with DNA Blood Mini Kit_ (Qiagen, Hilden, Germany). Quantity and quality were determined on the Nano-Drop_ ND-1000 spectrophotometer (ThermoScientific,Waltham, MA, USA). A panel of 571 genes, including genes known or predicted to be related with PIDs and/or immune regulation, was selected. 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 variant analysis

A TNGS panel, "TaGSCANv.2" (Targeted Gene Sequencing and Custom Analysis) was run, including 571 genes. Samples were prepared for sequencing using TruSight Rapid Capture with TruSight Inherited Disease Oligos (Illumina); the MT genome is covered at 1000x by the addition of MT oligos during enrichment (IDT). Samples were sequenced to at least 2.5Gb on an Illumina MiSeq with TruSeq v3 reagents, yielding paired 250 nucleotide reads. Alignment, variant calling, and analysis was performed as previously described. Variant analysis was confined to coding and splice variants with a minor allele frequency (MAF) of 1% or less in the CMH internal database.

WES was performed in 18 cases. Nucleotides (37.7 million) of exons (the exome) were enriched 44-fold from genomic DNA from the patients and sequenced to an average, uniquely aligned coverage of 135-fold.

§ Sanger sequencing

Selected variants predicted to be damaging, along with consistent genotype-phenotype correlation were validated by Sanger sequencing using standard protocols, in both the proband and parents. gDNA was PCR-amplified using GoTaq polymerase (Promega) and specific primers (primer sequences available upon request). Amplicons were bi-directly sequenced using the BigDye Terminator version 1.1 cycle sequencing kit and an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies).

Exons 1 to 25 of STAT1 and their flanking intron sequences were amplified by PCR with specific oligonucleotide primers (available on request) and sequenced with the Applied Biosystems Big Dye terminator kit v1.1 (AB Foster City California) and an ABI Prism 3130xl Analyzer (Applied Biosystems). For each GOF STAT1 mutation, the prediction scores of 5 algorithms — SIFT,S1 Polyphen2,S2 Mutation Taster, LRT,S3 and PhyloPS4— and a newly designed prediction score combining all five algorithms, rPred were used to predict the likely impact of an amino-acid substitution on STAT1 protein structure and function. Higher scores indicate a higher probability of the mutation being deleterious, and the maximum score is 1.

IKBKG-specific primers were used to evaluate the full cDNAwith the following primer sets: forward, 5#-CCCTTGCCCTGTTGGATGAATAGGC-3#; reverse, 5#-AGGCGGGAGAGGAAAGCGCAGACTG-3#; and forward, 5#-AAGCTGGCCCAGTTGCAGGTGGCCT-3#; reverse, 5#-AGGTGGCATCCCAGTTGTGG-3#.

The eight exons of EDA-1 and EDA-2 were amplified through PCR using the following primer sets: 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#.

The exons 2 and 3 of the PRF1 coding region were amplified using standard polymerase chain reaction (PCR) conditions. PCR products were recovered from 1.5% agarose with a size marker, purified using the QIAquick Gel extraction kit (Qiagen, Hilden, Germany) and sequenced in an automatic ABI 377 DNAsequencer (Applied Biosystems, Foster City, CA).

§ PCR and quantitative real-time PCR analysis

Total RNA from patient samples and controls was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. RNA was reverse transcribed by Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany).

The qPCR reactions were performed in duplicate. The amplification of the cDNAs was performed using the SYBR Green and analyzed with the Light Cycler480 (Roche Applied Systems, Germany).

Total RNA was extracted from PBMC and fibroblasts left unstimulated or stimulated with TNFα, IL-1, LPS, I:C, and IFNγ. Total RNA was extracted from IL-2 activated NK cell left unstimulated or stimulated with IL-2 (100ng/ml) or IL-15 (100ng/ml) for 24 h using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. For reverse transcription polymerase chain reaction (RT-PCR) experiments, 1 μg of DNase-treated total RNA was used to synthesize the first strand of cDNA by the Improm-II Reverse Trascription System (Promega, Madison, WI, USA). For the RT-PCR analysis, Assays-on-DemandTM Products and Taqman Master Mix from Applied Biosystems were used according to the instruction manual to analyze CCL2, CCL3, IL-1, IL-6, CCl5, CXCl9, CXCL10, ICAM-1, STAT1, IFNγ, IL2RA,SOCS1, SOCS3 and GAPDH, gene expression.

§ Mutagenesis assay

eGFP STAT1 WT vector (Addgene) was used to obtain mutated STAT1 form carrying L351F and L400V variants. Mutations were generated by site direct mutagenesis QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies).

§ Plasmid transfection

For the complementation of patients' fibroblasts with plasmids carrying wild-type MYD88, fibroblasts were incubated with a mixture containing Lipofectamine 2000 reagent (Invitrogen

#52887) and plasmids. This complex was then removed and fibroblasts were incubated for 18 hours in Dulbecco modified Eagle medium supplemented with 10% FBS before stimulation.

U3C cells were seminated into 96-well plates (1 X 10^4/well) and transfected with 100 ng/well reporter plasmids and plasmids carrying alleles of STAT1 (L351F and L400V) or a mock vectorwith Arrest-in (Thermo Scientific). After 6 h, cells were transferred into medium containing 10% FBS and cultured for 24h.

§ Luciferase reporter assay

U3C cells seminated into 96-well plates (1 X 10^4/well) and transfected with 100 ng/well reporter plasmids and plasmids carrying alleles of STAT1 (L351F and L400V) were stimulated with IFNγ at different concentration (10, 100, 1000 UI/ml), and IL-27 (100 ng/ml) for 16h. Luciferase production was assesed with Dual-Glo luciferase assay system (Promega) and normalized with respect to Renilla luciferase activity. Data are expressed as fold inductions with respect to unstimulated cells.

§ Western blot

For Western blot analysis, cytoplasmic extracts were resolved on 8% polyacrylamide and subjected to immunoblots by standard procedures. Nitrocellulose membranes were first blocked for 1 h at room temperature in TBST containing 5% BSA and then incubated overnight at 4 °C with specific primary Abs in the same buffer. NK cells were cultured in IL-2 (1200U/ml)-containing medium, and then starved, in order to reduce basal phosphorylation.

Cells were then coltured for 16 hours at 37°C in RPMI containing 2% serum left unstimulated or stimulated with IFN-α (Prepotech, 10000U/ml for 30 minutes); IL-2 (Prepotech, 100ng/ml for 12 minutes); IL-15 (Prepotech, 50ng/ml for 12 minutes); and IL-21 (Peprotech, 50ng/ml for 15 minutes). Following stimulation, cells were placed on ice and washed twice with cold PBS. Protein was extracted by extraction buffer (RIPA Buffer Millipore) containing protease, phosphatase inhibitors (Thermo Scientific) and EDTA (Thermo Scientific), and maintained on ice for 15 minutes with occasional mixing. Insoluble material was removed by centrifugation at 12000 x g for 15 minutes at 4°C. Protein lysates (5mcg per sample) were subjected to SDS PAGE separation on 10% BIS-Tris gel (NuPage Life Technologies) and proteins were transferred to a polyvinylidene difluoride membrane (GE Healthcare) and immunoblotted with primary antibody anti-pSTAT1-Tyr-701, anti-pSTAT5-Tyr-694 (both Cell Signaling Technology, Danvers, MA, USA), anti-STAT5, anti-STAT1 (BD Transduction Laboratory) and anti-β-actin or tubulin (Sigma-Aldrich, St. Louise, MO, USA) as loading control. Detection was carried out using horseradish peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich) and revealed using the enhanced chemioluminescence system (Euroclone LITEAblot Extend) according to the manufacturer's instructions. All images were captured with UVP Biospectrum AC Imaging System. Densitometric analysis was performed using the Gel Pro version 3.2 Analyzer Software.

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.

§ Proliferative assay

Cell proliferation was analyzed by the thymidine incorporation assay. For the evaluation in vitro of proliferative response to mitogens of PMBC, cells were stimulated with phytohaemagglutinin (PHA; 8 μg/ml), concanavalin A (ConA; 8 μg/ml), pokeweed (PWM, 10 μg/ml) (Difco Laboratories), phorbol-12-myristate-13-acetate (PMA; 20 ng/ml) and ionomycin (0.5 mM) (Sigma Chemical Co). CD3 cross-linking (CD3 X-L) was performed by precoating tissue culture plates with 1 and 0.1 ng/ml purified anti-CD3 monoclonal antibody (Ortho Diagnostic). Cells were plated in triplicate at 1x105 viable cells/well in 96-well plates (BD Biosciences), in 200 μl of complete medium for 4 days. Cultures were pulsed with 0.5μCi 3H-thymidine for 8 hours before harvesting and the incorporated radioactivity measured by scintillation counting.

To analyse cellular proliferation of NK-cell lines derived from peripheral blood of patients and healthy donors, we detected intracellular Ki-67 protein, after 48h stimulation with IL-2 and IL-15. Polyclonal NK-cell lines were permeabilized with saponin solution (0.1% in PBS) at 4°C for 20 minute. Then cells were incubated with MoAb Ki-67 as primary anti-body (IgG1, mouse anti-human, 1/40 dilution; Dako, Copenhagen, Denmark) for one hour at 4°C, to detect nuclear proliferation antigen. The secondary antibody used was PE-conjugated isotype-specific goat anti-mouse (Southern Biotechnology, Birmingham, AL) and was

incubated for 30 min at 4°C, then cells were re-suspended in 500 µl of PBS solution for flow cytometry analysis.

§ Flow cytometry analysis

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−), IgMmemory B cells (CD3−CD19+CD24+IgM+CD27+), and switched memory B cells (CD3−CD19+CD24+IgM−CD27+).

Percentage of CD4+IL-17A+ (TH17) and CD4+IFN- γ + (TH1) cells was evaluated following PMA plus Ionomycin stimulation for 6 h. For the evaluation of CD4+IL-17A+ and CD4+IFN-γ+ cell development, CD4+ cells were separated from the patient and a healthy control by positive selection using human CD4 microbeads, and then cultured with anti-

CD28, anti-CD3 XL, IL-6, IL-1β, TGF-β1, IL-23 for 6 days. After 6 days cells were splitted and cultured for further 6 days with the addition of IL-2.

For flow cytometric evaluation of STAT1 phosphorilation, cells were left unstimulated or stimulated with IFN-γ (40000U/ml for 30 minutes), IL-2 (100ng/ml for 12 minutes) IL-15 (50ng/ml for 12 minutes) and were stained simultaneously using a fluorescein isothiocyanate (FITC)-conjugated mouse anti-CD3 IgG mAb and a R-Phycoerythrincyanin5.1 (PerCP-Cy5.5)-conjugated mouse anti-CD56 IgG mAb. Cells were fixed and permeabilized, according to the BD protocol (Protocol III), and stained with phycoerythrin (PE)-conjugated mouse anti-pSTAT1-Tyr-701 and anti-pSTAT5-Tyr-694 IgG mAbs (BD Pharmigene) or isotype-matched mAb PE (BD Bioscence). Cells were acquired using a FACSCalibur flow cytometer (BD Bioscence) and analyzed by the FlowJo version 7.5 Software (TreeStar). Cells were fixed and permeabilized, according to the BD protocol (Protocol III), and stained with phycoerythrin (PE)-conjugated mouse anti-pSTAT1-Tyr-701, anti-pSTAT5-Tyr-694, anti-STAT1, anti-STAT5 IgG mAbs (BD Pharmigene) or isotype-matched mAb PE (BD Bioscence).

§ Analysis of NK cell cytotoxicity, degranulation and IFN-γ production

For degranulation assay against erythroleukemia K562 human cell line, PBMCs derived from patients and from healthy donors were incubated with or without 100 U/mL rh-IL-2 (Proleukin; Chiron) at 37°C overnight. Then, samples were co-incubated with target cells at a ratio of 1:3 (effector:target K562), in a final volume of 200 μl in round-bottomed 96 -well plates at 37°C and 5% CO2 for 3 hours in culture medium supplemented with anti-CD107a-

PE mAb. After 1h of co-incubation, GolgiStop (BD Biosciences Pharmingen, San Diego, CA, USA) was added at a 1:100 dilution. Surface staining was done by incubating the cells with anti-CD3, anti-CD14, anti-CD20 and anti-CD56 mAbs for 30 min at 4°C. Cells were washed and analyzed by flow cytometry (FACSCanto, Becton Dickinson). Analysis of NK cells was made on CD56+CD3-CD14-CD20- gated cells. NK cytotoxic activity was investigated in a 4-h 51Cr release assay with 5 • 103cells/well at a final ratio of 1:20 (T:E) with unstimulated or IL-2-activated NK cells against K562 cells as target.

To detect intracellular production of IFN-γ, PBMCs derived from patients and from healthy donors were incubated overnight at 37°C with IL-15 (20 ng/ml, Peprotech), or IL12 (20 ng/ml, Peprotech) and IL18 (100ng/ml, Peprotech) combined. Cells were then washed, fixed and permeabilized with Cytofix/Cytoperm kit (BD Bioscience Pharmingen). IFN-γ production was detected by subsequent intracellular staining with anti-IFN-γ-PE (BD Bioscience Pharmingen) upon gating on CD56+CD3-CD14-CD20- cells. For both degranulation and IFN-γ expression experiments, the percentage of positive cells was calculated subtracting the baseline CD107a or IFN-γ expression in controls cultures in the absence of stimuli (target cells or cytokines).

Perforin expression on peripheral blood NK cells (CD3-CD56+) was detected by intracellular staining after fixation and permeabilization with ΔG9 mAb (Becton-Dickinson Biosciences, Oxford, UK) and cytofluorimetric analysis.

§ Chromatin Immunoprecipitation (ChIP) assays

ChIP experiments were performed as previously described with minor modifications. Briefly, after stimulation with the appropriate cytokine, polyclonal NK cell lines were fixed using 1 % formaldehyde for 6 min followed by 0.125 M glycine for other 6 min to stop the cross-linking reaction. After 3 washes with ice-cold PBS, nuclear extract were prepared from 3.5x106 cells. Chromatin was then sheared by sonication and immunoprecipitated with 4 μg anti-STAT1 (sc-346) or 4 μg anti-STAT5 (sc-835) (Santa Cruz Biotechnology). To establish the background levels of ChIP experiments, the precipitation signal was quantified also at the promoter of prolactin (PRL). The coimmunoprecipitated material was subjected to qPCR analysis using the following promoter specific primers (purchased from Life Technologies): IL2RA forward: 5'-TTCCACAGTTCTGAGAAAGGTG-3'; IL2RA reverse: 5'-CATGGCAAGGGTTTATGAGG-3'; IFIT1 forward: 5'-GGCAGCAATGGACTGATGTTC-3'; IFIT1 reverse: 5'-GGAAACCGAAAGGGGAAAGTG-3'; and PRL forward: 5'-AGGGAAACGAATGCCTGATT-3'; PRL reverse: 5'-GCAGGAAACACACTTCACCA-3'. Data from qPCR were expressed as percentage over input DNA and are displayed as means ± SEM.

§ In vitro B cell proliferation and plasma cell differentiation

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 × 105 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 IgMantibodies (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.

§ Cytogenetic analysis

Karyotype was performed by standard GTG banding at 550 bands resolution (ISCN 2009). Array-CGH was performed with a 44 K whole-genome oligonucleotide microarray Agilent

Technologies (Santa Clara, CA) following the manufacturer protocol. Array data were visualized using the Genome Workbench standard edition ver. 5.0 Agilent Technologies (Santa Clara, CA) and compared with the human genome reference sequence hg19 (Feb. 2009).

§ Statistical analysis

All results are shown as the mean ± DS. Clinical features were compared in Chi-squared or Mann-Whitney U tests, as appropriate. Variables with a p-value < 0.05 were considered to be significant. SPSS Version 21 was used for the analysis (SPSS Inc., Chicago, Illinois). Survival curves were plotted as a function of age, by the Kaplan-Meier method. Continuous variables are expressed as the median and range, to reflect the population distribution.

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SUMMARY

Primary immunodeficiency disorders (PIDs) represent a heterogeneous group of inherited disorders characterized by poor or absent function in one or more components of the immune system, that result in chronic, recurrent and life-threatning infections if not promptly diagnosed and treated. Traditionally, PIDs are classified according to the component of the immune system that is primarily disrupted: innate or adaptive immunity, the latter comprising antibody deficiencies and combined immunodeficiencies. In the last 20 years, thanks to the progress in molecular technologies, a remarkable improvement of the knowledge in the field of PIDs, concerning both their etiopathogenesis mechanisms and clinical features, has been observed. Nowadays about 300 forms of well-characterized PIDs have been identified underliyng complex phenotype which encompass a wide spectrum of clinical features ranging from recurrent bacterial infections to other unusual manifestations, such as autoimmune disorder, cancer susceptibility, allergy and autoinflammation. In many cases, children affected with novel forms of PIDs also show extrahematopoietic alterations, leading to complex phenotypes characterized by a functional impairment of organs different from immune system. Advances in next generation DNA sequencing (NGS) allowed new gene identification of several forms of PIDs of unknown cause making genetic identification of immunodeficiency syndromes more efficient. Only in the last two years, 34 new gene defects have been identified. In this context, my PhD program has been focused to the study of some Immunological disorders, in order to identify "*Novel strategies in the approach to primary immunodeficiencies to discover new pathogenic mechanisms and complex clinical phenotype*". Particularly, I followed a first project focused on the novel insight in the

diagnosis and management of primary immunodeficiencies aimed at the characterization of novel aspect of the pathogenesis and treatment of already known immunodeficiency, diagnosed conventionally or through Next Generation Sequencing. In particular, I studied the role of Myd88 deficiency, identified through Targeted Next generation sequencing, in the pathogenesis of the immunological and clinical features observed in a patient who had an atypical presentation characterized by chronic Yersiniosis and granuolomatous lymphadenitis, in absence of pneumococcal infections. On this topic, I contributed to the description of the case and to the planning of the experiments aimed at demonstrating the defect of TLRs signaling and the rescue of the function after the tranfection of plamids containing WT Myd88 in the patient fibroblasts. The paper describing this patient has been accepted for the publication as *Letter* on *Journal of Allergy and Clinical Immunology*. I also followed another project aimed at defining the broad spectrum of clinical manifestations caused by STAT1 gain of function mutation and at defining the role of STAT1 gain of function mutation in the pathogenesis of the clinical manifestation caused by mutations in this gene other than chronic mucocutaneus candidiasis. Moreover, my research effort has been devoted to the definition of the role of T-independent B-cell immunity in susceptibility to infections from encapsulated bacteria in Hypoidrotic Ectodermal Dysplasia with immununodeficiency (HED-ID). I also participated to the description of skin and skin annexa abnormalities associated to PIDs, which represent alarm signs that should lead the clinician to consider a deeper immunological assessment. I gave a contribution to better define the functional role of FOXN1 transcription factor in the T-cell ontogeny. Eventually, I also

studied rare genetic syndrome involving immune system paying a particular attention to SCID, hemophagocytic lymphoistiocitosis (HLH) and Di George Syndrome (DGS).

CURRICULUM VITAE

DR. GIULIANA GIARDINO

PERSONAL INFORMATION

Giuliana Giardino

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Sex Female | Date of birth 08/07/1986 | Nationality Italian

EDUCATION AND TRAINING

04/2014–Present

PhD in “Human Reproduction, Development and Growth”

Unit of Immunology, Department of Translational Medical Sciences at the Federico II University of Naples, Naples (Italy)

Ph.D. student at the Doctoral Course in " Human Reproduction, Development and Growth ", Director prof Claudio Pignata, with a research project focused on the study of novel insight in the diagnosis and management of primary immunodeficiencies aimed to the characterization of novel aspect in the pathogenesis and treatment of already known immunodeficiencies, diagnosed conventionally or through Next Generation Sequencing.

01/2014–06/2014

Research fellow in Pediatric Immunology

Unit of Pediatric Immunology, Department of Pediatrics, Spedali Civili, University of Brescia, Brescia (Italy)

Diagnosis and treatment of the main primary immunodeficiencies including XLA, CVID, CGD, HIGM, WHIM, SCID due to Cernunnos mutation, autosomal recessive Hyper IgE Syndrome (DOCK8 mutation), Chronic mucocutaneous candidiasis (STAT 1 gain of function mutation), Leukocyte adhesion deficiency, Mendelian susceptibility to mycobacterial disease;

Evaluation of the main first and second level laboratory tests (Ig levels, Ig subclasses, response to polysaccharidic antigens, B- and T-cell immunophenotype, TRECs, NBT and DHR tests, proliferation assays); Study of NK cell and cytolytic function

07/2012–Present

Residency in Pediatrics

Department of Translational Medical Science at the Federico II University of Naples, Napoles (Italy)

03/2012

Degreee in Medicine and Surgery

Federico II University of Naples, Napoles (Italy)

Thesis internship at the Unit of Immunology, Department of Pediatrics of the Federico II University of Naples, Naples, Italy, focused on the study of a human model of athymia (Nude/SCID syndrome) through the characterization of peripheral and tissue lymphocytes in order to identify alternative extrathymic sites of T-cell ontogeny in the absence of the thymus.

Degree in Medicine and Surgery, with a thesis entitled: "Dimostrazione nell'uomo di linfopoiesi T extratimica timo indipendente". Vote: 110/110 cum laude.

PERSONAL SKILLS

Mother tongue(s): Italian

Other language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
Inglese	B2	B2	B2	B2	C1

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

Communication skills

Predisposition to work in a team of 5-6 persons by collaborating each other in a friendly manner developed during my Residency and Ph.D. period.

Organisational / managerial skills

Capacity to design a scientific project. Capacity to coordinate students in their practice also by follow them in the preparation of the thesis.

Digital competence

SELF-ASSESSMENT				
Information processing	Communication	Content creation	Safety	Problem solving
Proficient user	Independent user	Proficient user	Basic user	Basic user

Digital competences - Self-assessment grid

- Use of Endnote 7.0 to format and add references to a manuscript.
- Use of Word, graphical softwares such as Power Point, Publisher, Photoshop for Mac and PC, statistical software, such as Excel, GraphPad Prism and software to elaborate images.
- The above cited competence was acquired during the Ph.D. period spent at the Department of Translational Medical Sciences where she also learned to write a scientific paper and to create images and graphics and to prepare lessons, seminars and posters for meetings (See the attached list of scientific production).

ADDITIONAL INFORMATION

Member of the following scientific society

"Young member", of the European Society for Immunodeficiencies (ESID);

Member of the Società Italiana di Pediatria (SIP)

Reviewer for international Journals

International Review of Immunology

Scientific Interests

Major fields of scientific interests are as follows:

Molecular analysis of genes whose mutations are responsible for congenital immunodeficiencies, such as STAT1, NEMO, MYD88, Perforin, GP91phox and FOXN1.

T-cell ontogeny process in humans. In particular, the focus is to identify possible extrathymic sites of T-cell differentiation by comparing lymphocytic phenotypes from Nude/SCID and DiGeorge patients that represent two different models of human athymia.

Primary Immunodeficiencies: definition of novel therapeutical strategies for the treatment of Ataxia-Teleangiectasia. In particular, I participated in the activity of Prof. Pignata group during a clinical trial based on the use of bethametasone to improve neurological function in the affected patients.

Publications

1. Dotta L, Scomodon O, Padoan R, Timpano S, Plebani A, Soresina A, Lougaris V, Concolino D, Nicoletti A, **Giardino G**, Licari A, Marseglia G, Pignata C, Tamassia N, Facchetti F, Vairo D, Badolato R. Clinical and immunological data of nine patients with chronic mucocutaneous candidiasis disease. Data Brief. 2016 Feb 23;7:311-5.
2. Dotta L, Scomodon O, Padoan R, Timpano S, Plebani A, Soresina A, Lougaris V, Concolino D, Nicoletti A, **Giardino G**, Licari A, Marseglia G, Pignata C, Tamassia N, Facchetti F, Vairo D, Badolato R. Clinical heterogeneity of dominant chronic mucocutaneous candidiasis disease: presenting as treatment-resistant candidiasis and chronic lung disease. Clin Immunol. 2016 Mar;164:1-9.
3. **Giardino G**, Gallo V, Somma D, Farrow EG, Thiffault I, D'Assante R, Donofrio V, Paciolla M, Ursini MV, Leonardi A, Saunders CJ, Pignata C. Targeted next-generation sequencing revealed MYD88 deficiency in a child with chronic yersiniosis and granulomatous lymphadenitis. J Allergy Clin Immunol. 2015 *In Press.*
4. **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. 2015 Oct 15. doi: 10.1111/pai.12496. [Epub ahead of print].

5. **Giardino G**, Cirillo E, Gallo V, Esposito T, Fusco F, Conte MI, Quinti I, Ursini MV, Carsetti R, Pignata C. B cells from nuclear factor kB essential modulator deficient patients fail to differentiate to antibody secreting cells in response to TLR9 ligand. Clin Immunol. 2015 Aug 22;161(2):131-135.
6. 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 Jul 31. doi: 10.1111/nyas.12849. [Epub ahead of print].
7. 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 Mar 16. [Epub ahead of print].
8. Palamaro L, Romano R, Fusco A, **Giardino G**, Gallo V, Pignata C. FOXN1 in organ development and human diseases. Int Rev Immunol. 2014 Mar;33(2):83-93. doi: 10.3109/08830185.2013.870171. Epub 2014 Jan 17.
9. Cirillo E, **Giardino G**, Gallo V, Puliafito P, Azzari C, Bacchetta R, Cardinale F, Cicalese MP, Consolini R, Martino S, Martire B, Molinatto C, Plebani A, Scarano G, Soresina A, Cancrini C, Rossi P, Digilio MC, Pignata C. Intergenerational and intrafamilial phenotypic variability in 22q11.2 deletion syndrome subjects. BMC Med Genet. 2014 Jan 2;15:1. doi: 10.1186/1471-2350-15-1.
10. Cirillo E., Gallo V., **Giardino G.**, Pignata C. Immunodeficienze primitive: cosa c'è di nuovo. Prospettive in Pediatria 43:14-22, 2014.
11. **Giardino G**, Cirillo E, Maio F, Gallo V, Esposito T, Naddei R, Grasso F, Pignata C. Gastrointestinal involvement in patients affected with 22q11.2 deletion syndrome. Scand J Gastroenterol. 2014 Mar;49(3):274-9. doi: 10.3109/00365521.2013.855814. Epub 2013 Dec 18.
12. Conte MI, Pescatore A, Paciolla M, Esposito E, Miano MG, Lioi MB, McAleer MA, **Giardino G**, Pignata C, Irvine AD, Scheuerle AE, Royer G, Hadj-Rabia S, Bodemer C, Bonnefont JP, Munnich A, Smahi A, Steffann J, Fusco F, Ursini MV. Insight into IKBKG/NEMO locus: report of new mutations and complex genomic rearrangements leading to incontinentia pigmenti disease. Hum Mutat. 2014 Feb;35(2):165-77. doi: 10.1002/humu.22483. Epub 2013 Dec 12.
13. Montella S, Mollica C, Finocchi A, Pession A, Pietrogrande MC, Trizzino A, Ranucci G, Maglione M, **Giardino G**, Salvatore M, Santamaria F, Pignata C. Non invasive assessment of lung disease in ataxia telangiectasia by high-field magnetic resonance imaging. J Clin Immunol. 2013 Oct;33(7):1185-91. doi: 10.1007/s10875-013-9933-y. Epub 2013 Aug 24.
14. Romano R, Palamaro L, Fusco A, **Giardino G**, Gallo V, Del Vecchio L, Pignata C. FOXN1: A Master Regulator Gene of Thymic Epithelial Development Program. Front Immunol. 2013 Jul 12;4:187. doi: 10.3389/fimmu.2013.00187.
15. **Giardino G**, Veropalumbo C, Ruggiero G, Naddei R, Rubino V, Udhayachandran A, Cirillo E, Gallo V, Poggi V, De Fusco C, Pignata C. Phenotypic characterization and outcome of paediatric patients affected with haemophagocytic syndrome of unknown genetic cause. Br J Haematol. 2013 Sep;162(5):713-7. doi:10.1111/bjh.12421. Epub 2013 Jun 29.
16. van de Vosse E, van Dissel JT, Palamaro L, **Giardino G**, Santamaria F, Romano R, Fusco A, Montella S, Salerno M, Ursini MV, Pignata C. The R156H variation in IL-12Rβ1 is not a mutation. Ital J Pediatr. 2013 Feb 14;39:12. doi:10.1186/1824-7288-39-12.
17. Spagnuolo MI, Russo G, **Giardino G**, Caiazzo MA, Cirillo E, Ranucci G, Guarino A, Martire B, Vecchione R, Di Matteo G, Postorivo D, Pignata C. Chronic granulomatous disease with gastrointestinal presentation: diagnostic pitfalls andnovel ultrastructural findings. J Investig Allergol Clin Immunol. 2012;22(7):527-9.
18. Gallo V, **Giardino G**, Capalbo D, Palamaro L, Romano R, Santamaria F, Maio F, Salerno M, Vajro P, Pignata C. Alterations of the autoimmune regulator transcription factor and failure of central tolerance: APECED as a model. Expert Rev Clin Immunol.

2013 Jan;9(1):43-51. doi: 10.1586/eci.12.88.

19. Loffredo L, Carnevale R, Sanguigni V, Plebani A, Rossi P, Pignata C, De Mattia D, Finocchi A, Martire B, Pietrogrande MC, Martino S, Gambineri E, **Giardino G**, Soresina AR, Martino F, Pignatelli P, Violi F. Does NADPH oxidase deficiency cause artery dilatation in humans? Antioxid Redox Signal. 2013 Apr 20;18(12):1491-6. doi: 10.1089/ars.2012.4987. Epub 2012 Dec 7.
20. Capalbo D, De Martino L, **Giardino G**, Di Mase R, Di Donato I, Parenti G, Vajro P, Pignata C, Salerno M. Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy: insights into genotype-phenotype correlation. Int J Endocrinol. 2012;2012:353250. doi: 10.1155/2012/353250. Epub 2012 Oct 22.
21. **Giardino G**, Fusco A, Romano R, Gallo V, Maio F, Esposito T, Palamaro L, Parenti G, Salerno MC, Vajro P, Pignata C. Betamethasone therapy in ataxia telangiectasia: unraveling the rationale of this serendipitous observation on the basis of the pathogenesis. Eur J Neurol. 2013 May;20(5):740-7. doi: 10.1111/ene.12024. Epub 2012 Nov 4.
22. Capalbo D, **Giardino G**, Martino LD, Palamaro L, Romano R, Gallo V, Cirillo E, Salerno M, Pignata C. Genetic basis of altered central tolerance and autoimmune diseases: a lesson from AIRE mutations. Int Rev Immunol. 2012 Oct;31(5):344-62. doi: 10.3109/08830185.2012.697230.
23. Palamaro L, **Giardino G**, Santamaria F, Romano R, Fusco A, Montella S, Salerno M, Ursini MV, Pignata C. Interleukin 12 receptor deficiency in a child with recurrent bronchopneumonia and very high IgE levels. Ital J Pediatr. 2012 Sep 19;38:46. doi: 10.1186/1824-7288-38-46.
24. Montella S, Maglione M, **Giardino G**, Di Giorgio A, Palamaro L, Mirra V, Ursini MV, Salerno M, Pignata C, Caffarelli C, Santamaria F. Hyper IgM syndrome presenting as chronic suppurative lung disease. Ital J Pediatr. 2012 Sep 19;38:45. doi: 10.1186/1824-7288-38-45.
25. Palamaro L, **Giardino G**, Santamaria F, Ramenghi U, Dianzani U, Pignata C. Altered regulatory mechanisms governing cell survival in children affected with clustering of autoimmune disorders. Ital J Pediatr. 2012 Sep 12;38:42. doi: 10.1186/1824-7288-38-42.
26. Cirillo E, Romano R, Romano A, **Giardino G**, Durandy A, Nitsch L, Genesio R, Di Gregorio E, Cavalieri S, Abate G, Del Vecchio L, Brusco A, Pignata C. De novo 13q12.3-q14.11 deletion involving BRCA2 gene in a patient with developmental delay, elevated IgM levels, transient ataxia, and cerebellar hypoplasia, mimicking an A-T like phenotype. Am J Med Genet A. 2012 Oct;158A(10):2571-6. doi:10.1002/ajmg.a.35556.
27. Romano R, Palamaro L, Fusco A, Iannace L, Maio S, Vigliano I, **Giardino G**, Pignata C. From murine to human nude/SCID: the thymus, T-cell development and the missing link. Clin Dev Immunol. 2012;2012:467101. doi: 10.1155/2012/467101.
28. Palamaro L, Vigliano I, **Giardino G**, Cirillo E, Aloj G, Romano R, Pignata C. SCID-like phenotype associated with an inhibitory autoreactive immunoglobulin. J Investig Allergol Clin Immunol. 2012;22(1):67-70.
29. Aloj G, **Giardino G**, Valentino L, Maio F, Gallo V, Esposito T, Naddei R, Cirillo E, Pignata C. Severe combined immunodeficiences: new and old scenarios. Int Rev Immunol. 2012 Feb;31(1):43-65. doi: 10.3109/08830185.2011.644607.
30. Broccoletti T., Aloj G., **Giardino G.**, Pignata C. Ataxia teleangiectasia: molecular biology, diagnosis and treatment.Nova Publishers. pp 29-45, 2012. ISBN: 978-1-61942-867-6 BOOK CHAPTER.
31. Pignata C, **Giardino G**. Immunodeficiency diagnosis: a Mondrian or Pollock scenario? Blood. 2011 Nov 24;118(22):5714-6. doi: 10.1182/blood-2011-09-379065.
32. **Giardino G**, Aloj G, Cirillo E, Capalbo D, Maio F, Salerno M, Franzese A, Pignata C. Intergenerational anticipation of disease onset in people with multiple autoimmune syndrome. Diabetes Res Clin Pract. 2011 Nov;94(2):e37-9. doi:

10.1016/j.diabres.2011.07.022. Epub 2011 Aug 17.

33. Palamaro L., Guarino V., Scalia G., Antonimi D., DeFalco L., Vigliano I., Fusco A., Vitiello L., **Giardino G.**, Caterina M., Del Vecchio L., Ambrosio L., Pignata C. 3-dimensional poly(3-caprolactone) scaffold containing skin-derived fibroblasts and keratinocytes supports in vitro HSCs differentiation in T-lineage-committed cells.Advanced School in Primary ImmunoDeficiency Disease Chicago 18-19 Maggio 2011 (oral abstract)
34. Broccoletti T, Del Giudice E, Cirillo E, Vigliano I, **Giardino G**, Ginocchio VM, Bruscoli S, Riccardi C, Pignata C. Efficacy of very-low-dose betamethasone on neurological symptoms in ataxia-telangiectasia. Eur J Neurol. 2011Apr;18(4):564-70. doi: 10.1111/j.1468-1331.2010.03203.x. Epub 2010 Sep 14.
35. Fusco A, Vigliano I, Palamaro L, Cirillo E, Aloj G, Piscopo G, **Giardino G**, Pignata C. Altered signaling through IL-12 receptor in children with very high serum IgE levels. Cell Immunol. 2010;265(1):74-9. doi: 10.1016/j.cellimm.2010.07.005. Epub 2010 Jul 21.

Meeting Abstracts and Communications

1. **G. Giardino**, E. Cirillo, V. Gallo, R. D'Assante, M. Paciolla, G. Ruggiero, M. V. Ursini, R. Carsetti, A. Puel, C. Pignata: Chronic Mucocutaneous Candidiasis, Recurrent Herpetic Infections and Suppurative Eyelid Infections in a Patient Carrying a Novel Gain-of-Function Mutation in the STAT1 DNA-Binding Domain. 16th Biennial Meeting of the European-Society-for-Immunodeficiencies; 10/2014
2. Gallo V., Cirillo E., **Giardino G.**, D'Assante R., Spennato P., Cinalli G., Pignata C. Intrathecal amphotericin B therapy in a patient with X-linked chronic granulomatous disease and refractory cerebral invasive aspergillosis. ESID Meeting. J Clin Immunol. 34: 282, 2014.
3. **Giardino G.**, Naddei R., Cirillo E., Gallo V., Esposito T., Fusco F., Quinti I., Ursini M.V., Carsetti R., Pignata C. TLR9 signaling in patients with ectodermal dysplasia and immunodeficiency associated with Nuclear Factor Essential Modulator (NEMO) mutations. ESID Meeting. J Clin Immunol. 34: 403, 2014.
4. Cirillo E., Gallo V., **Giardino G.**, Galasso G., Romano R., D'Assante R., Genesio R., Baldini A., Nitsch L., Pignata C. DiGeorge-like syndrome in a child with a 3p12.3 deletion involving miRNA-4273 born to a diabetic mother.ESID Meeting. J Clin Immunol. 34: 440, 2014.
5. Quarantelli M, **Giardino G**, Prinster A, Aloj G, Carotenuto B, Cirillo E, Marsili A, Salvatore E, Del Giudice E, Pignata C. Steroid treatment in Ataxia-Telangiectasia induces alterations of functional magnetic resonance imaging during prono-supination task. Eur J Paediatr Neurol. 2013 Mar;17(2):135-40. doi: 10.1016/j.ejpn.2012.06.002. Epub 2012 Jul 2.
6. Cirillo E., **Giardino G.**, Puliafito P., Ariganello P., Azzari C., Cardinale F., Consolini R., Martino S., Plebani A., Scarano G., Soresina A.R., Cancrini C., Digilio M.C., Pignata C. Intergenerational familial phenotypic variabilità in 22q11.2 subjects: a multicentral study within the Italian Network for Primary Immunedeficiencies (IPINet). J Clin Immunol. 32:195-196, 2012.
7. Cirillo E., Romano R., **Giardino G.**, Anne D., Maio F., Gallo V., Di Gregorio E., Cavalieri S., Abate G. Del Vecchio L., Brusco A., Pignata C. Elevated IgM levels in a patient with de novo 13q12.3q14.11 deletion , mimicking an A-T like phenotype. J Clin Immunol. 32:211-212, 2012.
8. Veropalumbo C., **Giardino G.**, Cirillo E., Gallo V., Maio F., Esposito T., Naddei R., Grasso F., Poggi V., De Fusco C., Pignata C. Retrospective analysis of 21 pediatric patients affected with hemophagocytic syndrome of unknown genetic cause: clinical features and outcome. J Clin Immunol. 32: 212-213, 2012.

9. Broccoletti T., Del Giudice E., Cirillo E., **Giardino G.**, Vigliano I., Ginocchio V.M., Bruscoli S., Riccardi C., Pignata C. Estimate the minimum therpeutically effective dosage of short-term therapy with Betamethasone on neurological symptoms in patients affected with Ataxia-Telangectasia. Clin Immunol. 135: 314, 2010.
10. Cirillo E., Fusco A., Vigliano I., Palamaro L., Aloj G., **Giardino G.**, Gallo V., Maio F., Valentino L., Cosentini E., and Pignata C. Severe combined immunodeficiency phenocopy associated with an inhibitory autoreactive immunoglobulin. XIVth Meeting for the European Society for Immunodeficiencies. Instanbul 6-9 October 2010.
11. **Giardino G.**, Cirillo E., Aloj G., Valentino L., Maio F., Gallo V., Franzese A., Pignata C. Intergenerational comparison of clinical phenotype in patients affected with Hypex-like syndrome (HLS). XIVth Meeting for the European Society for Immunodeficiencies. Instanbul 6-9 October 2010.
12. Broccoletti T., Del Giudice E., Cirillo E., **Giardino G.**, Vigliano I., Ginocchio V. M., Bruscoli S., Riccardi C., Pignata C. Estimate the minimum therpeutically effective dosage of short-term therapy with Betamethasone on neurologicaal symptoms in patients affected with Ataxia-Telangectasia. First CIS North American Primary Immune Deficiency National Conference. Philadelphia 20-23 maggio, 2010.
13. Broccoletti T., Del Giudice E., **Giardino G.**, Cirillo E., Aloj G., Quarantelli M., Prinster A., Bruscoli S., Riccardi C., Pignata C. The effects of betamethasone therapy on neurological functions in A-T patients. The International Ataxia-Telangiectasia Workshop 2010. Los Angeles 11-14 aprile, 2010
14. Aloj G., Cirillo E., Broccoletti T., Valentino L., **Giardino G.**, Maio F., Ginocchio V.M., Del Giudice E., Pignata C. Miglioramento dei sintomi neurologici dell'Atassia-Teleangiectasia durante trattamento con betametasone: ricerca della dose minima efficace.13° Congresso Nazionale "Incontri Pediatrici Normanni", Aversa 11-12 Dicembre, 2009.
15. **Giardino G.**, Broccoletti T., Cirillo E., Maio F., Valentino L., AlojG., Gallo V., Pignata C. Uso dell'ipoclorito di sodio per uso topico nella dermatite atopica resistente ai comuni trattamenti.13° Congresso Nazionale "Incontri Pediatrici Normanni", Aversa 11-12 Dicembre, 2009.
16. Maio F., Cirillo E., Broccoletti T., **Giardino G.**, Valentino L., Aloj G., Gallo V., Pignata C. Effetto del rituximab sulla citopenia associata alla sindrome linfoproliferativa autoimmune (ALPS) refrattaria alle comuni terapie.13° Congresso Nazionale "Incontri Pediatrici Normanni", Aversa 11-12 Dicembre, 2009.
17. Broccoletti T., Valentino L., Cirillo E., Maio F., **Giardino G.**, Aloj G., Gallo V., Pignata C. Un caso atipico di sindrome da Iper-IgM. 13° Congresso Nazionale "Incontri Pediatrici Normanni", Aversa 11-12 Dicembre, 2009.

Awards

1. Palamaro L., Guarino V., Scalia G., Antonini D., **Giardino G**., De Falco L., Vigliano I., Fusco A., Missero C., Del Vecchio L., Ambrosio L., Pignata C. 3-dimensional poly(3-caprolactone) scaffold containing skin-derived fibroblasts and keratinocytes supports in vitro HSCs differentiation in T lineage-committed cells. Advanced School in Primary Immune Deficiency. Chicago, 18-19 Maggio, 2011.

Application to grant proposal

1. Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale (PRIN) 2015-2016, with a project entitled: "Targeted next generation sequencing as an approach to patients with severe forms of congenital immunodeficiencies".
2. Jeffrey Modell Foundation Application 2016, with a project entitled: "In vitro development of medullary thymic epithelial cells (mTECs) obtained by reprogramming technology to support HSC differentiation into fully mature single-positive T cells on a 3D thymic organoid".