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XXXIV CICLO DEL DOTTORATO DI RICERCA IN SCIENZE DELLA RIPRODUZIONE E DELLO SVILUPPO

CLINICAL, IMMUNOLOGICAL, GENETIC AND TRANSCRIPTOME CHARACTERIZATION OF IMMUNE DYSREGULATIONS

Settore scientifico-disciplinare: **MED/38**

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RIASSUNTO

Le immunodeficienze primitive (PID) includono più di 350 malattie monogeniche, che intaccano lo sviluppo e la funzione del sistema immunitario. Negli ultimi vent'anni, è stato notato che alcune di queste patologie si presentano con prevalenza di sintomi infiammatori e autoimmune correlati a disregolazione immune piuttosto che a infezioni ricorrenti. Le immunodeficienze da disregolazione possono condividere alcune caratteristiche con le più comuni condizioni infiammatorie e autoimmuni, rendendo difficile dire quando sia giustificato richiedere un esame immunologico e genetico. I peculiari quadri clinici che supportano un sospetto di PID da disregolazione, possono includere due distinti gruppi di sintomi: un fenotipo autoimmune (per cui abbiamo definito il criterio di arruolamento A) o un fenotipo allergico (per cui abbiamo definito il criterio di arruolamento B). I soggetti che rispettano questi criteri sono stati provvisoriamente considerati come affetti da *Undefined Dysregulatory Disorders* (UDDs), fino a quando non verrà evidenziata una causa genetica.

Lo scopo del mio progetto di dottorato è quello di valutare se i profili immunologici e genetici possano contribuire a migliorare gli approcci diagnostici e i piani terapeutici nei pazienti affetti da UDD. Il confronto tra casi con o senza una causa monogenica accertata, in cui sono coinvolte note vie di segnalazione patogenetiche, può aiutare nel definire il significato patologico dei dati immunologici e trascrittomici. In aggiunta, vogliamo capire se casi di UDD con genetica negativa possano essere raggruppati sulla base di una strategia di analisi combinata, in base alle loro similitudini con malattie monogeniche definite.

In questi 3 anni abbiamo arruolato 30 pazienti, 15 che ricadevano nel criterio A, 12 nel criterio B e 3 che li rispecchiano entrambi. Sono state eseguite le seguenti indagini: sottopopolazioni linfocitarie, analisi genetiche, saggi funzionali e studi di trascrittomica. Attraverso questa strategia combinata di analisi, a 7 pazienti è stata diagnostica una delle seguenti malattie monogeniche: *LRBA* e *CTLA4 deficiency* in 3 pazienti con fenotipo autoimmune, IPEX (mutazione nel gene *FOXP3*) in un paziente con fenotipo allergico e mutazioni nei geni *FASLG*, *STAT1* e *TNFRSF13B* in 3 pazienti che rispecchiano le caratteristiche di entrambi i fenotipi.

Rispetto ai 23 pazienti rimasti, questi 7 casi con genetica positiva, che quindi possono non essere più considerati come UDD, tendono ad avere dei quadri clinici tra i più complessi. In più, le combinazioni di sintomi osservate in questi pazienti possono essere riscontrate anche nei pazienti rimanenti con genetica negativa, suggerendo quindi l'ipotesi che possano

condividere alterazioni patogenetiche nelle stesse vie di segnalazione, le quali possono essere rilevanti per la scoperta di nuove cause genetiche o per la proposta di trattamenti.
Inoltre, i pazienti in cui è stata identificata una malattia monogenica conosciuta possono trarre beneficio dal trattamento con terapie di precisione.

ABSTRACT

Primary immunodeficiencies (PID) include more than 350 monogenic diseases, which affect the development and function of the immune system. In the last twenty years, it has been noticed that some of these pathologies occur with predominant inflammatory or autoimmune symptoms arising from immune dysregulation, rather than recurrent infections. Dysregulatory immune deficiencies may share clinical features with more common autoimmune or inflammatory conditions, making it difficult to tell when an immune and genetic workup is warranted. Typical clinical pictures supportive of a dysregulatory PID can include two distinct sets of symptoms: an autoimmune phenotype (for which we defined recruitment criteria A) or an allergic phenotype (for which we defined recruitment criteria B). Subjects meeting these criteria with Undefined Dysregulatory Disorders (UDDs) until a genetic cause is highlighted.

The aim of my PhD project is to evaluate if immunological and gene expression profiles may contribute to develop improved diagnostic approaches and therapeutic directions in patients with UDDs. Comparison between cases with or without a detectable monogenic cause, in which definite pathogenic pathways are involved, can help to define the pathologic significance of immunological and transcriptomic data. Moreover, we want to understand if genetic negative UDDs cases can be grouped based on a combined analysis strategy, according to their similarity with distinct monogenic disease.

In these 3 years we enrolled 30 patients, 15 complying criteria A, 12 complying criteria B and 3 sharing both criteria. We analyzed the immunophenotype by flow cytometry and performed genetic investigations, along with functional assays and transcriptome studies. According to this combined analysis strategy, 7 patients were diagnosed with a monogenic disorder, namely LRBA and CTLA4 deficiency in 3 patients with autoimmune phenotype, IPEX syndrome (*FOXP3* mutation) in 1 patient with allergic phenotype and defects of *FASLG*, *STAT1* and *TNFRSF13B* in 3 patients meeting selection criteria in both groups.

Compared with the remaining 23 patients, these 7 cases with a positive genetic cause, who may no longer be considered UDDs, tend to have more complex clinical pictures.

Furthermore, combinations of symptoms displayed by these patients can be recognized also in the remaining ones with negative genetic raising the question if they share the same pathogenic pathways, which could be relevant to the discovery of new genetic causes or to the proposal of mechanistic treatments.

Indeed, patients with identification of known monogenic disorders can receive a targeted therapy, in which precision therapies are available.

SUMMARY

1. INTRODUCTION

Primary immunodeficiencies (PIDs) include more than 350 monogenic diseases, related to dysregulation of immune system, which can result in infections, autoinflammation, autoimmunity, lymphoproliferation and/or risk of malignancy (1). Since the term PID brings to mind the lack of immune components resulting in risk of infections, many experts currently prefer to rely on the term "inborn errors of immunity" (IEIs) to underline the complexity and the heterogeneity of phenotypes due to immune defects (2).

The diagnosis of a primary immunodeficiency disease should be always considered in subjects with recurrence of serious infections, but in the last twenty years this paradigm has partly changed. It has been noticed that an increasing number of immune defects are associated with inflammatory or autoimmune features that may occur before infections or even in the absence of significant infectious risk (3, 4).

1.1 Discovery of primary immunodeficiencies in the second Half of the last century

The discovery of PIDs started more than 60 years ago, when the decrease of morbidity from infections, thanks to improved nutrition, hygiene and medicines, allowed noticing cases with unexpected recurrence of severe infections, often together with inflammatory and autoimmune manifestations. Since immunology was still a young discipline in the past century, knowledge on primary immunodeficiency diseases also provided crucial examples to understand how distinct components of the immune system work (3, 4), by describing the consequences of the defective function of specific immune cells or mechanisms. Data from human experience of the so-called "experiments of nature" were paralleled by advances in knowledge on animal models, mainly in mice.

The first PID described was X-linked agammaglobulinemia, also called Bruton's disease, in 1952, which is caused by mutations in the *BTK* gene which is involved in the B cells development and in the pathway of response to Toll-like receptor stimulation. Patients with Bruton's disease show recurrent respiratory and gastrointestinal infections, chronic diarrhea and gastrointestinal manifestations resembling inflammatory bowel disease (IBD) (1, 5). Moreover, an increased production of autoantibodies can paradoxically be observed in these patients, due to hypomorphic *BTK* mutations resulting in enhanced maturation and survival of autoreactive B cells.

Even if it the concepts of “antibody” and the recognition of the importance of humoral and cellular immunity date back to the end of the ‘800s, only in the 50s the description of a group of disorders with defects in adaptive immune response have been described as antibody deficiency syndromes (defective humoral branch of immunity) and combined immunodeficiencies (CIDs, reflecting defective cellular branch of immunity) (6).

On one hand, defective antibody production diseases, the most common type of immunodeficiencies, were associated with increase susceptibility mostly to respiratory bacterial infections but might also cause abscesses in the skin or other organs, urinary tract infections and inflammatory/autoimmune complaints like arthritis, often combined with diarrhea and autoimmune cytopenia (3, 6). These syndromes depend on a variegated spectrum of development maturation and function of B cells as in the case of Bruton’s agammaglobulinemia. The most representative group of B cells disorders is common variable immunodeficiency (CVID), often defined by normal count of B lymphocytes and almost always by the reduction of serum concentration of one or more immunoglobulin isotypes that implies recurrent pulmonary infections in addiction to autoimmune and granulomatous disease and gastrointestinal complications (3, 6, 7).

On the other hand, CIDs have been defined as T-cell disorders, which comprised a heterogeneous group of diseases with impaired development and/or function of lymphocytes associated with reduction in immune cell count and antibody production (3, 5, 6). Typical examples of CIDs are listed in the following table 1.

CID disorder	**Clinical presentation**	**Molecular involvement**
Wiskott-Aldrich syndrome	thrombocytopenia; eczema; recurrent bacterial and viral infections; autoimmune manifestations like arthritis and neutropenia	mutation in *WASp* gene that affects the actin cytoskeleton and T cell signaling pathways
Ataxia telangiectasia	chronic sinopulmonary disease; cerebellar ataxia; small, dilated blood vessels of the eyes and skin; malignancy; hypogammaglobulinemia; lymphopenia	mutation in *ATM* gene causes uncontrolled cellular growth and division
DiGeorge syndrome (described in 1965)	hypoparathyroidism; cardiac abnormalities; facial	impaired development of thymus, according to

	dysmorphisms; infections	moderate T cell deficiency, and parathyroid glands
Hyper-IgE syndrome (HIES) (described in 1966)	chronic dermatitis; recurrent and severe lung infections, skin infections; bone fragility; elevated levels of serum IgE	impared of Th17 function which also results in development of autoimmunity
SCID	severe and recurrent infections; diarrhea; rash	lack of T and/or B cells

Table 1: first examples of CIDs (1, 3, 6)

Severe combined immunodeficiencies (SCIDs) are the most severe PIDs, presenting by definition in the first year of life with various combinations of symptoms, ranging from severe infections of bacterial, viral, or fungal origin, to inflammatory and autoimmune manifestation, such as enteropathy, chronic diarrhea, dermatitis and autoimmune cytopenia (1, 5, 6, 8). Patients with SCID may be characterized either by the lack of T cells with normal B cell count ($T^- B^+$) or by the lack of both lymphocyte populations ($T^- B^-$) (6, 8). In almost all patients with SCID the severe defect of T lymphocyte maturation is witnessed by the lack of recent thymic emigrant lymphocytes (RTE), (fig.1) which represents the thymic output of naive cells with a proper T cell receptor (TCR) assembly. Interestingly, TCR assembly implies the cut out of a piece of DNA from the genome, which will remain circularized but not replicant within the cell (T cell receptor excision circle, TREC), providing a reliable molecular marker for RTE that is being now exploited in newborn screening for SCIDs (3, 5, 8).

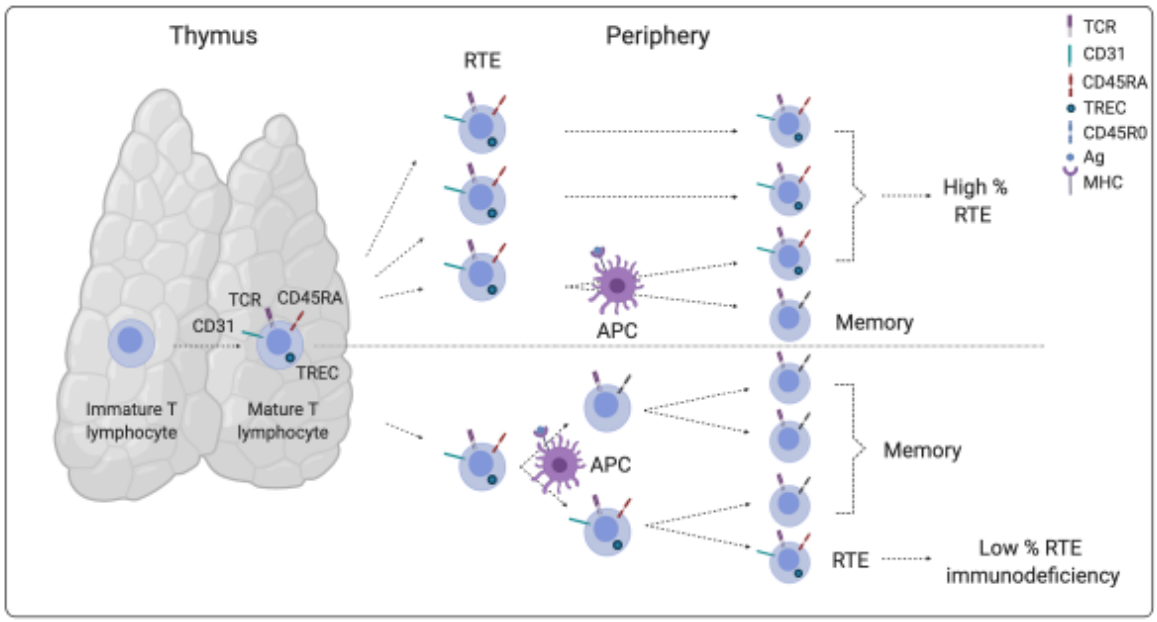

Fig.1: RTE in healthy people and in patients with immunodeficiency like SCID (8).

Although phagocytes were first immune cells to be characterized in the last decades of the 19th century by Élie Metchnikoff (9, 10), only between the 50s and 70s the importance of innate immunity was highlighted by the discovery of disorders with defective phagocyte function presenting with severe infections in early life (bacterial and fungal skin, respiratory and internal organ infections). These disorders include severe congenital neutropenia (SCN), leukocyte adhesion deficiency (LAD), characterized by impaired trafficking of leukocytes and Chronic Granulomatous Disease (CGD), characterized by failure of oxidative burst in phagocytes. SCN is the most severe form of neutropenia, characterized by severe and often life-threatening infections since their first months of life (11). LAD, in turn, includes 3 distinct forms: LAD1 where leukocytes do not adhere stably with the endothelium due to the mutation in *ITGB2* gene that encodes CD18 (β_2 integrin) and therefore cannot migrate to infection/inflammation site; LAD2 is due to mutations in the ligand of E-selectin and LAD3 caused by mutations involved in integrin signaling (3, 6). CGD, which is usually characterized by systemic autoimmune manifestations, granulomatous colitis and severe or recurrent infections often associated with certain bacterial and fungi (1, 3) is caused by mutations in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex that is involved in the production of microbicidal compounds and activates lytic enzymes to kill engulfed pathogens (1) (fig.2).

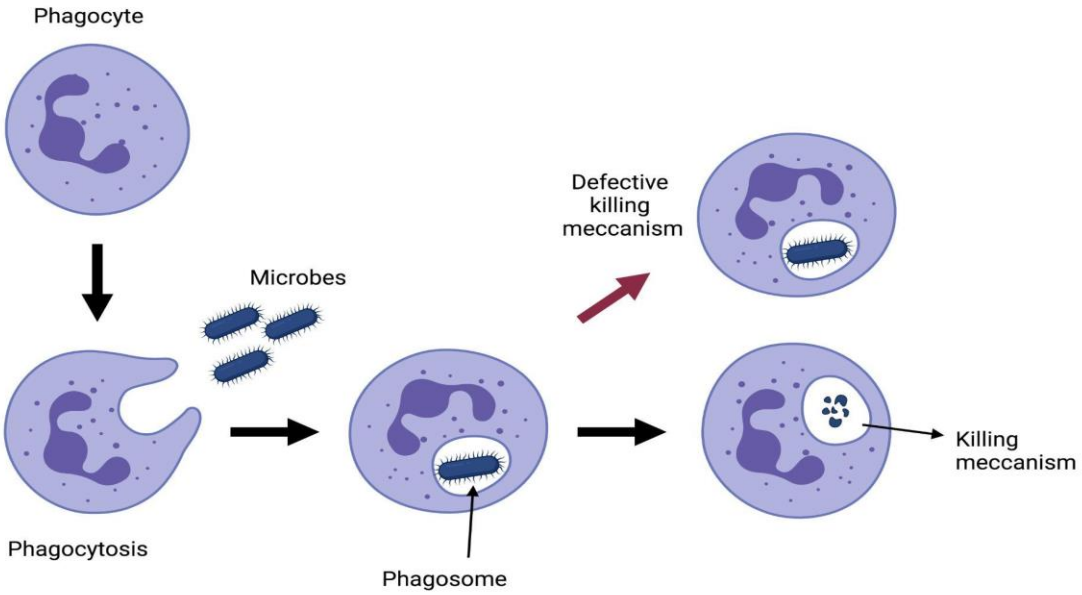

Fig.2: mechanism of phagocytosis and killing of microbes (figure created with BioRender.com).

1.2 The theory of clonal selection and the enigma of immune tolerance

Immune defects involving the production of antibodies, the development of lymphocytes and the function of phagocytes helped understanding the importance of specific immune mechanisms in the fight to various kinds of microbes. However, crucial immunological questions remained unanswered without an informative monogenic example. Indeed, a fundamental step forward in deciphering how the immune system works came in the 50s with the Burnet's theory of clonal selection, postulating that each lymphocyte undergoes somatic recombination of DNA generating a unique TCR specificity that can be clonally amplified upon antigen recognition (12, 13). Of note, this theory implied the existence of mechanisms of control preventing the maturation or activation of cells with self-reactive receptors, leading to postulate the existence of central and/or peripheral tolerance. The concept of tolerance gained more importance in 1953 when Peter Medawar, inspired by Burnet's study, succeeded in inducing graft tolerance in a mouse infused at birth with lymphocytes derived from the spleen of a mouse of another strain. Indeed, a few weeks after this treatment the first mouse received and tolerated a skin graft from the second one. In this way Medawar demonstrated the validity of Burnet's theory of self/non-self discrimination underlying that a successful transplantation can be possible and well tolerated if the recipient's immune system is overcome (14).

But to that moment the basis for immunological tolerance remained elusive, until 1995 when Sakaguchi discovered a new T cell subpopulation with regulatory properties, called regulatory T cells (Treg) in an opportune murine model. Starting from the observation that both in humans and in mice the $CD4^+$ T cells population are directly involved in organ-specific and systemic autoimmune diseases, he wanted to understand what mechanisms of immunological tolerance regulate the $CD4^+$ self-reactive T cells and demonstrated that elimination of $CD4^+$ subpopulation expressing CD25 (IL-2 receptor α-chains), later called regulatory T (Treg), leads to the spontaneous onset of various cellular and humoral autoimmune manifestations. Of note, the adoptive transfer of $CD4^+$ $CD25^+$ cells to the animal led to reversal of the autoimmune phenotype, suggesting that this cell fractions contains Tregs (15-17). The presence of autoantibodies implies that also the self-tolerance of B cells is broken and probably due to stimulatory signals rescuing autoreactive B cells from apoptosis (15-17).

In a normal setting, regulation of activated cells to prevent excessive responses can be obtained by Treg with the help of various accessory molecules like CTLA4, which is constitutively express and play a critical role in the suppression function of $CD25^+$ $CD4^+$ T cells (fig.3) (15, 16, 18). If healthy mice are treated with anti-CTLA4 monoclonal antibody over a limited period, the number of Treg is not reduced but the block of CTLA4 implicates the onset of autoimmune manifestations similarly to those associated with defective Treg (16, 18).

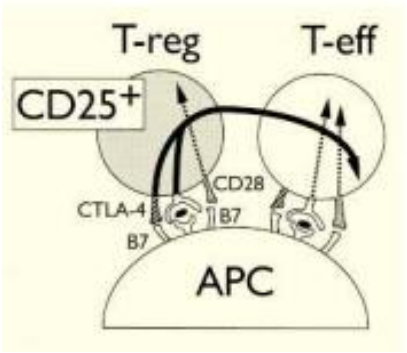

Fig.3: mechanism of inhibition of T effector by Treg due to the influence of CTLA4 (16).

This imbalance of immune system following removal of $CD25^+$ $CD4^+$ T cells cannot easily associate to T lymphocytopenia and to the consequent increase of susceptibility to infection, because this subpopulation constitutes only the 5-10% of peripheral $CD4^+$ T cells, but the decline of them is enough to break natural self-tolerance and to induce autoimmune disorders (16).

According to these results, Sakaguchi hypothesized that some autoimmune diseases in mice and in humans might arise from a deficit of the mechanism of immunological self-tolerance

and he postulated the possible existence of genetic disorders with autoimmunity correlated to reduced number or function of Treg (15, 16).

1.3 Understanding defective tolerance at the turn of the 20th century

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) has been the first disorder associated with defective function of the Treg population described by Sakaguchi. IPEX was described for the first time in 1982 in a family with many males affected (inherited X-linked recessive) with a combination of symptoms of intractable diarrhea, eczema, hemolytic anemia, diabetes mellitus and thyroid autoimmunity, without any specific hallmarks of any immunologic disorder previously described (19, 20) but it was associated with defective T cell tolerance only two decades later.

Causative mutations in the human gene *FOXP3* and in the mouse orthologue *Foxp3* were found to account respectively for IPEX syndrome and for a closely similar murine disorder, called scurfy mice, allowing to translate to human immunological lessons derived from studies in the animal (21, 22) (fig.4).

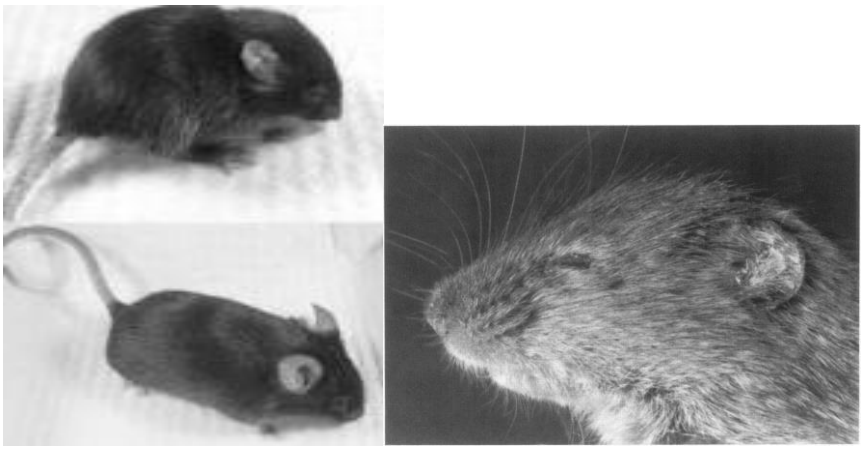

Fig.4: top left, scurfy mouse compared with normal mouse lower left: the former is smaller than latter with small ears and scaly tail. Lower right a zoom of squinted eyes and small crusted ears (22).

It was sure that the phenotype of mice was due to an immune regulatory defect correlated with $CD4^+$ T lymphocytes that show elevated levels of lymphocyte activation markers like CD69, CD25 and B7, but they are not constitutively activated, but rather are hyperresponsive to TCR stimulation, probably due to B7 high expression (21). The first consequence of this picture is an overproduction of cytokines, including IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ e TNF-α at

levels of mRNA and protein expression (21, 22). Taken together, these observations support a role of the scurfy gene in the regulatory mechanism of T cell functions (21). The gene involved in scurfy mice was identified in 2001. The protein encoded by this gene, called *Foxp3,* is a transcription factor, defined as a new member of forkhead/winged-helix family, whose forkhead domain, which links DNA, is lost due to the effect of the mutation in *Foxp3* in scurfy mouse (23). Almost simultaneously, in 2001, Bennet and Wildin (24, 25) found that human IPEX syndrome was due to mutations in *FOXP3,* the human orthologue of *Foxp3,* and a couple of years later it would became clear that that FOXP3 transcription factor has a crucial role in the function of regulatory T cells and immune tolerance (26-28) (fig.5)

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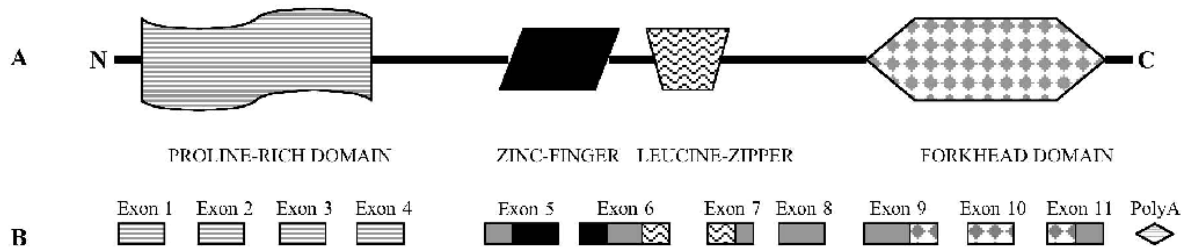


Fig.5: (A) Schematic structure of the FOXP3 protein with all domains. (B) Exon organization of the *FOXP3* gene, including the regions encoding each protein domain (29).

Moreover, the *FOXP3* expression level was measured in tissues and purified lymphocytes and the highest level was found in thymus, spleen and lymph nodes and in $CD25^{+}$ $CD4^{+}$ regulatory T cells respectively (29-31). The major expression of *FOXP3* in Treg and the observation in scurfy mouse that retroviral gene transfer of *Foxp3* into naive T cells confers a regulatory function on these ones, confirmed the *Foxp3* key role for development and function of Treg (28-31) (fig.6).

The requirement for intact FOXP3 protein for functional regulatory T cells was further confirmed by studies evaluating X-chromosome inactivation in peripheral blood of females heterozygous for *FOXP3* mutations. The study showed that cell with a regulatory T cells phenotype ($CD3^{+}CD4^{+}CD25^{high}$) always displayed a skewed X-inactivation profile, expressing the X-chromosome containing the wild type allele, whilst conventional T cells displayed a random X-inactivation pattern. These results confirmed: 1) that intact FOXP3 is necessary for the development and function of regulatory T cells; 2) that the presence of functional T regs is sufficient to regulate conventional T cells regardless of their activation of the healthy or diseased C-chromosome (32).

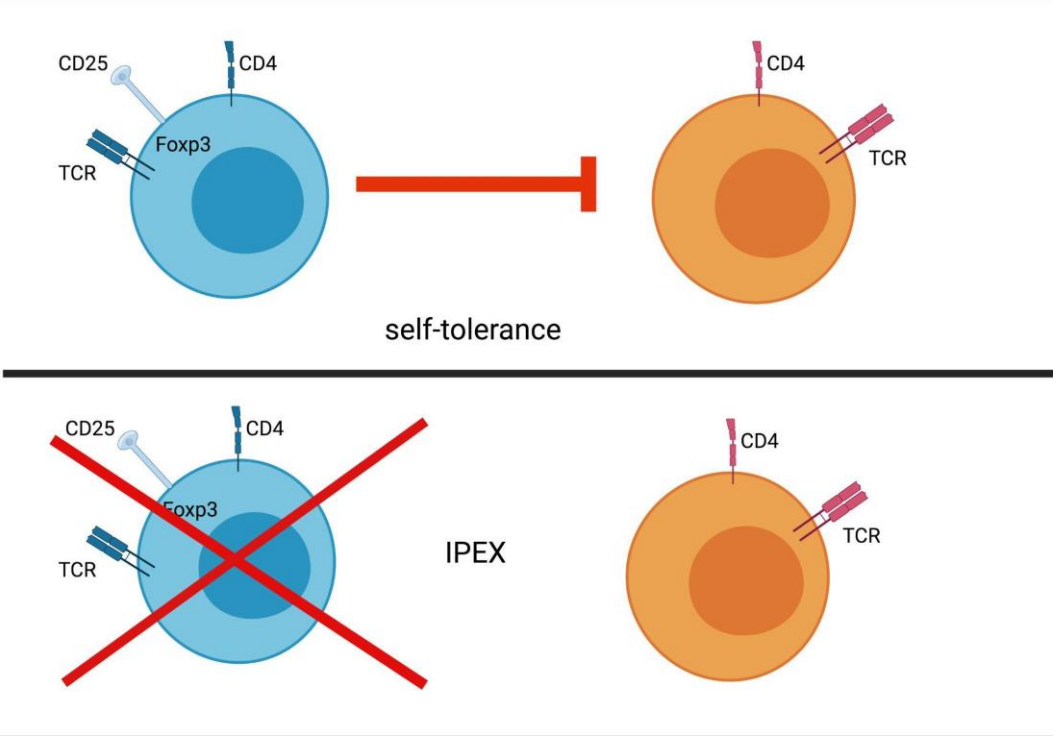


Fig.6: schematic view of self-tolerance against IPEX syndrome (figure created with BioRender.com)

The characterization of IPEX fueled new interest in other PIDs associated with immune dysregulation, like APECED, described in 1980, whose genetic cause was found in mutations of *AIRE* gene in 1997 (33, 34), and ALPS, described in 1967 (35) and whose genetic cause was described in 1995 due to mutations in the *FAS* gene (36). The finding that the APECED association with AIRE protein is involved in thymic tolerance to ectopic antigens came in 2002, the same year in which the regulatory function of the FOXP3 protein was proved (28, 37).

These findings prompted new research to understand dysregulatory features of PIDs, changing the paradigm of recurrent severe infections as the cause of main clinical manifestations raising the suspicion of a PID, and increasing the awareness of inflammatory or autoimmune symptoms as possible presenting complaints, even in the absence of infections (3, 4).

Indeed, considering the protean roles and the complex regulation of the immune system, it should not be surprising that many defects can result in a variable combination of infectious, inflammatory, autoimmune and neoplastic features.

In some cases, PIDs tend to affect multiple organs and systems, composing complex clinical pictures that overlap disorders of distinct medical specialties, and tend to fully manifest over the time, with the definition of typical clinical pictures only in adults. For this reason, they may initially suggest the diagnosis of common multifactorial disorders, rather than raise the

suspicion of a monogenic inborn disease. However, atypical features like a very early onset in childhood, multiorgan involvement and refractoriness to conventional therapies may prompt physicians to suspect a dysregulatory disorder.

There are some "red flags" that could help diagnose a PID in children with complex immune-mediated disorders:

1. early onset in childhood;
2. presence of multiple autoimmune and inflammatory manifestations;
3. lymphoproliferative manifestations: the presence of splenomegaly and/or lymphadenopathy in association with inflammatory or autoimmune diseases suggests an underlying inborn error of immunity;
4. recurrent infections in association with inflammatory or autoimmune diseases is rare in children, so it often suggests an immunological defect;
5. familiarity with autoimmunity;
6. specific laboratory abnormalities: hypo or hypergammaglobulinemia, leukopenia, hypereosinophilia, positivity of autoantibodies, positive interferon signature.

Improved diagnostics of monogenic immune disorders, together with the availability of medications acting on molecular disease-related mechanisms recently led to the development of precision therapies which can improve or correct the phenotype of some immunodeficiencies (38-40). Therefore, considering that effective treatments are now available for these immune disorders, it is crucial to consider the possibility of a primary immune defect in subjects presenting with clinical pictures suggestive of immune dysregulation, that could overlap distinct rheumatological, gastroenterological, endocrinological and dermatological/allergic disorders.

Some immunodeficiencies for which precision therapies are available are briefly summarized in table 2.

PID	Gene	Mechanism	Immune assessment	Clinics autoimmunity	Lympho proliferation	Infections	Therapies
APDS	*PIK3CD* *PIK3R1*	PI3K delta hyperactivation	hypogammaglobulinemia (IgA and IgG); reduced RTE; increased senescent CD8 T cells	IBD; diabetes; arthritis	lymphadenopathy splenomegaly	recurrent respiratory infections; herpes virus infections	HSCT; antibiotics rituximab and rapamycin; PI3Kδ inhibitors

STAT3 GOF	*STAT3*	STAT3 hyperactivation	hypogammaglobulinemia decrease NK cells; decrease memory B cells; decrease regulatory T cells	autoimmune cytopenia; diabetes; thyroiditis; arthritis	adenopathy; hepatomegaly; splenomegaly	herpes virus infections; fungal infections; bacterial infections; respiratory infections	JACK inhibitors
APECED	*AIRE*	decrease of negative selection of autoreactive T cells in thymus	autoantibodies; CD8+ effector T cells; FOXP3+ regulatory T cells	autoimmune hypoparathyroidism; Addison's disease		chronic Candida infection	hormone replacement therapy according to affected organs; Immunosuppressive therapies; rituximab
CTLA4 deficiency	*CTLA4*	defective switch off of lymphocyte activation	hypogammaglobulinemia increase of DNT; increase of regulatory T cells with reduced expression of FOXP3; decrease of CD19+ B cells and switched memory B	autoimmune cytopenia; hemolytic anemia and thrombocytopenia	splenomegaly; chronic lymphadenopathy; hepatomegaly	respiratory tract infections	sirolimus; abatacept; hematopoietic stem cell transplantation (HSCT)
LRBA deficiency	*LRBA*	defective switch off of lymphocyte activation	hypogammaglobulinemia increase of DNT; decrease of FOXP3+ regulatory T cells; decrease of CD19+ B cells and Natural Killer cells; increase of CD4+ and CD8+ memory T cells	autoimmune gastritis; autoimmune cytopenia; hemolytic anemia; IBD; Autoimmune enteropathy	splenomegaly; hepatomegaly	Respiratory infections	sirolimus; abatacept; hematopoietic stem cell transplantation (HSCT)
IPEX	*FOXP3*	failure of immune tolerance	loss of FOXP3+ T cells; increased of	autoimmune enteropathy; autoimmune hemolytic anemia;		skin infections	glucocorticoids; sirolimus; tacrolimus;

			Th2 and Th17 cells; autoantibodies; hypogammaglobulinemia (IgA, IgE)	autoimmune thrombocytopenia; autoimmune neutropenia; autoimmune thyroiditis; nephropathy; hepatitis			abatacept; hematopoietic stem cell transplantation (HSCT)
STAT1 GOF	*STAT1*	STAT1 hyperactivation due to increase STAT1 phosphorylation	low Th17 cells; low switched memory B cells; hypergammaglobulinemia (IgG)	mucocutaneous candidiasis; hypothyroidism; autoimmune cytopenia, hepatopathy; psoriasis	hepatomegaly; splenomegaly	fungal, viral and mycobacterial infections; skin infections; respiratory infections	antifungal treatment; antibiotic prophylaxis; JACK inhibitors
DADA2	*ADA2*	reduced activity level of the adenosine deaminase 2	hypogammaglobulinemia; increases macrophage release of TNF-α; upregulation of neutrophil activity; upregulation of pro-inflammatory cytokines; upregulation of type 1 interferon stimulated genes; aberrant B cell development and differentiation ; decrease in NK	vasculitis, immunodeficiency; autoimmune neutropenia; autoimmune cytopenia	splenomegaly; lymphadenopathy ; hepatomegaly	Verrucosis; herpes virus infections; increased susceptibility to infection with dsDNA viruses	anti-TNF treatment (etanercept, infliximab,adalimumab); high-dose of glucocorticoids; hematopoietic stem cell transplantation (HSCT); immunosuppressive drugs in isolated cases (mycophenolate, azathioprine, cyclosporine, rituximab, sirolimus, tacrolimus)
TNFAIP3 deficiency	*TNFAIP3*	excessive activation of NF-kB signalling	antinuclear and anti-DNA antibodies; increased production of interferons and proinflammatory cytokines	autoimmune cytopenias		recurrent respiratory and otorhinolaryngological infections	anti-TNF treatment; anti-IL1 treatment; glucocorticoid; colchicine

Table 2: Characteristics of pathologies for which precision therapies are available (29, 41-49).

In the last twenty-five years, 178 subjects received a diagnosis of inborn error of immunity due to mutations in about 59 distinct genes in IRCCS Burlo Garofolo hospital, highlighting the high genetic heterogeneity underpinning PIDs (fig.7) (8). This complexity, together with the rarity of PIDs, make it difficult to optimize protocols for diagnosis and treatment. The easy availability of genetic testing in recent years has only partially improved these procedures, as it opened new questions on the interpretation of results of uncertain significance and on the necessity of proper clinical and immunological correlations.

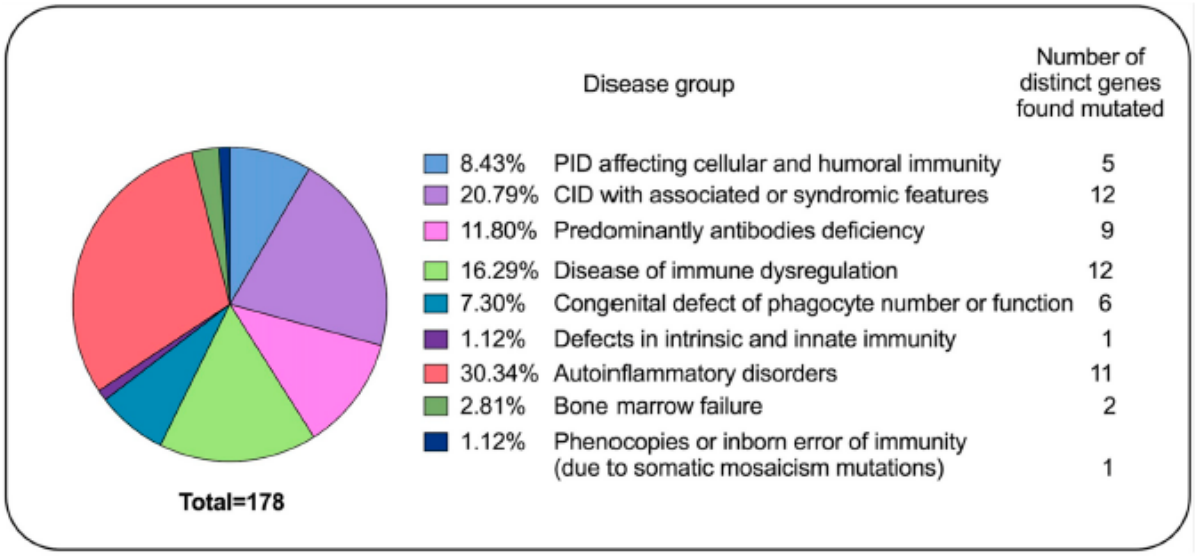


Fig.7: Primary immunodeficiency disease (PID) diagnosis in the last twenty-years in IRCCS Burlo Garofolo (8).

1.4 Genetic approach evolution

The race in search of variants related to immune dysregulation began by sequencing single genes linked to well characterized PIDs in patients with supportive clinical pictures. However, due to the rarity of these disorders and to their genetic heterogeneity underpinning immune dysregulation disorders, and thanks to the increased capabilities of genetic examinations with next generation sequencing (NGS), based single gene approaches had been replaced by multi-gene panels (8). Target gene panel is useful in cases with clinical phenotype that can overlap a narrow set of monogenic immunodeficiencies allowing higher sequences coverage and an easier first-line evaluation of results. However, for patients with the suspicion of an undefined PID, whole genome sequencing can be preferred, due to the possibility of identifying copy number variations, large deletions, and other genomic rearrangements. However, the consequent large production of data produced by such analyses may require further clinical and immunological investigations to clarify the significance of genetic results in genes not yet

associated with PIDs (8, 50). Furthermore, genetic studies may yield lots of variants of uncertain significance (VUS), requiring new immunologic studies and functional assays for a definite confirmation.

In most cases, gene panels or clinical exome sequencing can help to define a PID (8, 51).

Even after the identification of causative genes for several defects, laboratory immune evaluation remained crucial for diagnosis of many PIDs due to the prompt availability of the results and to the functional significance of assays that could reflect the severity of the underlying molecular defect. Flow cytometry immunophenotyping remains an essential exam in many PIDs, but it can result normal in some immunodeficiency, so that it cannot be used as the only tool to rule out the diagnosis of a PID (8, 52).

The great impact given by the NGS in the diagnosis of rare diseases has made it possible to characterize certain groups of diseases (8, 53) such as monogenic inflammatory bowel diseases (IBD), providing in some cases useful data for diseases stratification (53). In 2019, Lega et all enrolled 93 patients diagnosed with IBD and studied with NGS. 12 patients received a diagnosis of monogenic IBD that impacted patient therapy (53). This study approach explained a good strategy to tell when a genetic workup is warranted (fig.8):

- the study of single gene in cases with clinical or immunological characteristics specific for known monogenic defects;
- NGS should be preferred in patients with no specific phenotypes above all in early-onset diseases which are really suggestive of genetic defects.

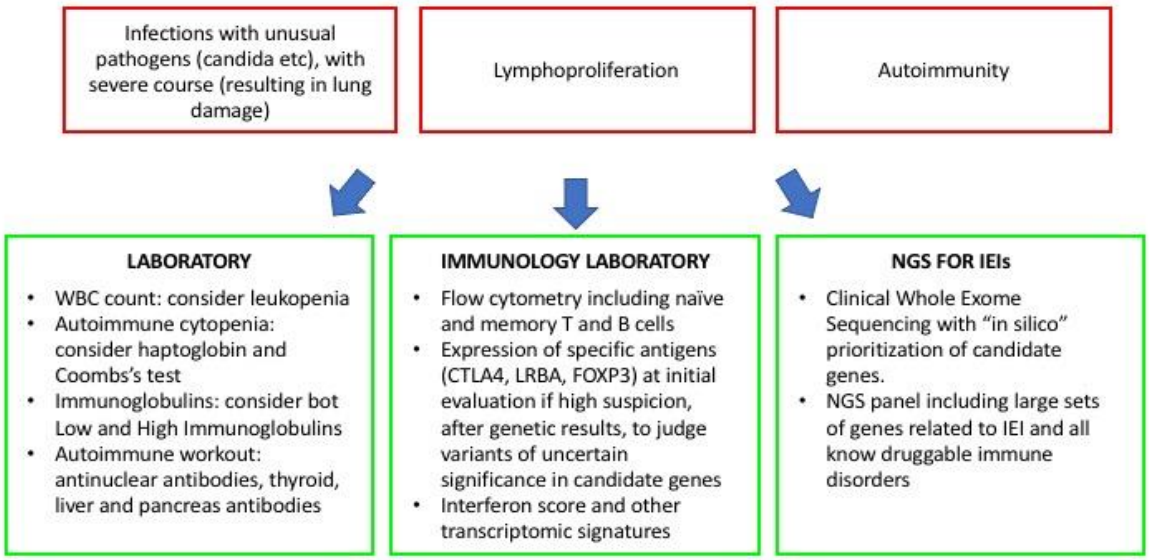


Fig.8: A simplified diagram for suspicion and diagnosis of druggable IEI in clinical practice.

More recently, the widespread use of molecular genetics greatly increased the capacity to identify PIDs, and correlations between genotypes and phenotypes have begun to be established (8, 54, 55). However, since the clinical phenotype of "druggable" PIDs may overlap with common rheumatologic or gastroenterological disorders, it is important to increase the awareness of the possible genetic diagnosis among diverse medical specialists with complex pathologists.

Dysregulatory immune defects may share clinical features with more common autoimmune or inflammatory conditions making it difficult to tell when an immune and genetic workup is warranted. Typical clinical pictures supportive of a dysregulatory PID can include two distinct sets of symptoms: an autoimmune phenotype or an allergic phenotype.

In this PhD project we defined two recruitment criteria which reflect the previous phenotypes and subjects meeting these criteria were considered as affected with Undefined Dysregulatory Disorders (UDDs) and we hypothesize that at least some of these subjects with UDD may have a monogenic disease affecting other genes involved in the mechanisms involved in immune regulation.

2. AIM

The aims of my PhD project are:

- to describe the proportion and characteristics of subjects with UDD that are eventually diagnosed with a definite monogenic PID;
- to compare clinical, immunological and expression profile between subjects with definite monogenic PIDs and those who remain without an underpinning genetic immune defect after proper evaluation;
- to discuss how immunological and expression profiles may impact on diagnostic approaches and therapeutic directions, with particular reference to cases without an underpinning genetic immune defect.

The main point of workflow:

1. clinical characterization of patients enrolled according to recruitment criteria;
2. definition of functional-genetic profiles of immune dysregulation diseases:
    a) to analyze lymphocyte subpopulations in flow cytometry;
    b) to measure the prevalence of monogenic diseases among subjects with UDD;
    c) to identify new genetic variants associated with defects of immune dysregulation through WES in complex and severe clinical pictures;
3. correlation between clinical and laboratory features:
    a) to describe the correlation between clinical and immunological phenotypes and genetic and functional data;
    b) to find clinical and laboratory features with potential predictive value for diagnosis and prognosis;
    c) elaborate advices to make immunological and genetic test in UDD cases.

3. MATERIAL & METHODS

3.1 Recruitment criteria

As discussed above, typical clinical pictures supportive of dysregulatory immunodeficiencies can include two distinct patterns of symptoms, respectively dominated by autoimmune or allergic phenomena. Thus, we elaborated inclusion criteria to enroll in the study children and adults who had onset of symptoms in pediatric age:

- recruitment criteria A associated with an autoimmune phenotype;
- recruitment criteria B associated with an allergic phenotype.

3.1.1 Criteria A

In this criteria we included patients with complex diseases characterized by autoimmune manifestations (gastrointestinal, hepatic, cutaneous, endocrine, hematological) accompanied by the presence of at least one of the following accessory symptoms: hypergammaglobulinemia, hypogammaglobulinemia, eosinophilia and lymphatic proliferation.

3.1.2 Criteria B

This criteria included 4 different possible scenarios:

1. suspicion of hyper-IgE syndrome (> 2000Ul/mL) with severe dermatitis and at least one of the following elements: recurrent infections, skeletal and joint disorders, recurrent pneumonia, pyogenic cutaneous abscesses;
2. severe idiopathic eosinophilia (>5000/ul) together with hypergammaglobulinemia and at least one of dermatitis, enteropathy and lymphatic proliferation;
3. severe dermatitis refractory to topical corticosteroids treatment;
4. food anaphylaxis refractory to desensitizing treatments.

For all patients defined as UDD we collected DNA stored at -20°C, RNA, serum and peripheral blood mononuclear cells (PBMC) appropriately stored at -80°C. Moreover, clinical and laboratory data have been reported in a structured database where patients have been anonymized.

All patients were enrolled after signing an informed consent approved by the Local Ethical Committee.

3.2 Immunophenotype

The lymphocytes subsets of enrolled patients have been analyzed by flow cytometry. For each patient, a heparinized peripheral whole blood sample was obtained, and surface staining of whole blood or of PBMC was performed with multicolour antibody panels (table 2) in order to evaluate different lymphocyte subpopulations:

- B cell subpopulations
  - naïve follicular B cells (Bnaive), defined as $CD45^{++}CD19^{+}IgD/IgM^{+}CD27^{-}$;
  - IgM B memory cells (Bmem), defined as $CD45^{++}CD19^{+}IgD/IgM^{+}CD27^{+}$;
  - switched memory B cells (Bswi), defined as $CD45^{++}CD19^{+}IgD/IgM^{-}CD27^{+}$;
  - transitional B cells/recent bone marrow emigrants (RBE), defined as $CD45^{++}CD19^{+}IgD/IgM^{+}CD10^{+}$;
- T cell subpopulations
  - recent thymic emigrants (RTE), defined as $CD45^{++}CD3^{+}CD4^{+}CD31^{+}CD45RA^{+}$;
  - double negative T cells (DNT), defined as $CD45^{++}CD3^{+}CD4^{-}CD8^{-}$TCR a/b^{+};
  - CD4/CD8 ratio;
  - senescent CD8 T cells, defined as $CD45^{++}CD3^{+}CD8^{+}CD57^{+}CD45RA^{+}$;
  - activated T cells, defined as $CD45^{++}CD3^{+}CD4^{+}CD69^{+}$ and as $CD45^{++}CD3^{+}CD4^{+}HLA^{-}DR^{+}$;
  - regulatory T cells (Treg) defined as $CD45^{++}CD3^{+}CD4^{+}CD25^{+}CD127^{-}$;
- adhesion molecules of granulocytes defined as $CD45dimCD18^{+}CD11b^{+}$
- B-lymphocytes defined as $CD3^{+}CD19^{+}CD86^{+}$

Samples were acquired with MACSQuant Analyzer 10 cytometer and analyzed with FlowLogic software.

Lymphocyte subpopulation	Antigen	Fluorochrome	Firm
LRTE	CD3	VioBlue	Miltenyi
	CD45	V500-C	BD
	CD16	Viobright515	Miltenyi
	CD56	Viobright515	Miltenyi
	CD31	PE	Miltenyi
	CD8	PerCP-Cy5.5	BD
	CD45RA	PE-Vio-770	Miltenyi
	CD4	APC	BD
	CD19	APC-Cy7	BD
DNT	CD3	VioBlue	Miltenyi
	CD45	V500-C	BD
	TCRgd	FITC	Miltenyi
	TCRab	PE	Miltenyi
	CD8	PerCP-Cy5.5	BD
	B220	PE-Vio770	Miltenyi
	CD4	APC	BD
	CD95	APC-Vio770	Miltenyi
PHENOTYPE B CELLS	CD21	VioBlue	Miltenyi
	CD45	V500-C	BD
	CD38	FITC	BD
	IgD	PE	Miltenyi

	IgM	PE	Miltenyi
	IgG	PerCP-Vio770	Miltenyi
	CD10	PE-Vio770	Miltenyi
	CD27	APC	Miltenyi
	CD19	APC-Cy7	BD
CD8/CD57	CD3	VioBlue	Miltenyi
	CD45	V500-C	BD
	CD4	FITC	Caltag
	CD57	PE	Miltenyi
	CD8	PerCP-Cy5.5	BD
	CD45RA	PE-Vio-770	Miltenyi
	CD27	APC	Miltenyi
Memory and activated T cells	CD3	VioBlue	Miltenyi
	CD45	V500-C	BD
	HLA-DR	FITC	Miltenyi
	CD127	PE	Miltenyi
	HLA A,B,C	PerCP-Vio770	Miltenyi
	CD69	PE-Vio770	Miltenyi
	CD4	APC	BD
	CD25	APC-Cy7	Biolegend
Granulocytes	CD45	V500-C	BD
	CD16	Viobright515	Miltenyi
	CD11b	PE	Miltenyi
	CD18	PE-Cy5	BD

	CD10	PE-Vio770	Miltenyi
	CD15	APC	Miltenyi
	CD11c	APC-Vio770	Miltenyi
CD86	CD3	Vio-Blue	Miltenyi
	CD19	APC-Cy7	BD
	CD86	PE	Biolegend

Table 2: Surface markers and corresponding antibodies that characterized lymphocytes subpopulation analyzed.

3.3 Genetic analysis

For all genetic analyses, DNA was extracted from a sample of peripheral whole blood in EDTA, quantified and stored at -20°C.

3.3.1 Targeted gene panels sequencing (TGPS) and Whole exome sequencing (WES)

Custom-made panels of genes responsible for immune dysregulation were sequenced by Next Generation Sequencing (NGS) on PGM-Ion Torrent platform (Thermo Fisher Scientific) (table 3 and 4).

CTLA4	*IL21*	*IL21R*	*LRBA*	*NFNB2*
PIK3CD	*PIK3R1*	*RAG1*	*TNFRSF13B*	*TNFRSF13C*

Table 3: panels of genes responsible for CVID.

TNFAIP3	*STAT1*	*DNASE2*	*DNASE1L3*	*TMEM173*	*PRKCD*
TREX1	*SAMHD1*	*IFIH1*	*DNASE1*	*ISG15*	*PTPN22*
CTLA4	*STAT4*	*TLR4*	*RORC*	*RC3H1*	

Table 4: panels of genes involved in Systemic lupus erythematosus (SLE) and Behcet syndrome.

The signal processing was analyzed by the Torrent SuiteTM software v5.12 and the output file was further annotated using an home made pipeline (Annovar).

WES analysis was made by an external service, Biodiversa (Rovereto, Italy), through Next Generation Sequencing (NGS) on Illumina platform.

In both analysis technique All variants found were further filtered based on the following criteria:

- type of mutation (missense, nonsense, frameshift and splicing about +/-10 nucleotides from the splice site);
- pathogenic prediction according to in silico prediction tools, such as CADD, Polyphen-2 and SIFT;
- minor allele frequency (MAF) (absent if never described, <0.02 if recessive inheritance model or <0.001 if dominant inheritance model), according to gnomAD database;
- damaging prediction according to the American College of Medical Genetics (ACMG) guidelines into "pathogenic," "likely pathogenic," or "variants of uncertain significance" (VUS).

Finally, databases like OMIM and Human Gene Mutation Database (HGMD) were used to analyze the clinical meaning to the variants and associate a specific disease phenotype.

Variants considered to be causative and correlated to the clinical phenotype were confirmed in Sanger sequencing and possibly in family segregation analysis.

3.3.2 Clinical exome

Sample of peripheral whole blood in EDTA was sent to the external service Invitae in San Francisco (USA), where 407 genes associated with PID were sequenced and tested for deletion/duplication (table 5). This diagnostic test evaluates variants (genetic changes)

combined with family history, clinical findings and other medical results. Informations about allele frequency of the variant are derived from a number of public sites that aggregate data from large-scale population sequencing projects, including ExAC, gnomAD and dbSNP.

ACD	*ACP5*	*ACTB*	*ADA*	*ADA2*	*ADAM17*	*ADAR*	*AICDA*	*AIRE*	*AK2*
ALG6	*ANGPT1*	*ANKZF1*	*AP3B1*	*AP3D1*	*ARHGEF1*	*ARPC1B*	*ASAH1*	*ATM*	*ATP6AP1*
B2M	*BACH2*	*BCL10*	*BCL11B*	*BLM*	*BLNK*	*BLOC1S3*	*BLOC1S6*	*BTK*	*C17orf62*
C1QA	*C1QB*	*C1QC*	*C1S*	*C2*	*C3*	*C5*	*C6*	*C7*	*C8A*
C8B	*C9*	*CARD11*	*CARD14*	*CARD8*	*CARD9*	*CARMIL2*	*CASP10*	*CASP8*	*CBL*
CCBE1	*CD19*	*CD247*	*CD27*	*CD3D*	*CD3E*	*CD3G*	*CD40*	*CD40LG*	*CD46*
CD55	*CD59*	*CD79A*	*CD79B*	*CD81*	*CD8A*	*CDC42*	*CDCA7*	*CEBPE*	*CFB*
CFD	*CFH*	*CFI*	*CFP*	*CHD7*	*CIB1*	*CIITA*	*CLCN7*	*CLPB*	*COL7A1*
COPA	*CORO1A*	*CR2*	*CSF2RA*	*CSF2RB*	*CSF3R*	*CTC1*	*CTLA4*	*CTPS1*	*CTSC*
CXCR2	*CXCR4*	*CYBA*	*CYBB*	*CYP27A1*	*DCLRE1C*	*DDX58*	*DEF6*	*DGAT1*	*DIAPH1*
DKC1	*DNAJC21*	*DNASE1L3*	*DNASE2*	*DNMT3B*	*DOCK2*	*DOCK8*	*DSG1*	*DTNBP1*	*DUOX2*
EFL1	*EIF2AK3*	*ELANE*	*EPG5*	*ERBIN*	*ERCC2*	*ERCC3*	*ERCC6L2*	*EXTL3*	*FADD*
FANCA	*FANCB*	*FANCE*	*FANCF*	*FANCI*	*FANCL*	*FAS*	*FASLG*	*FAT4*	*FCHO1*
FERMT1	*FERMT3*	*FOXI3*	*FOXN1*	*FOXP3*	*FPR1*	*G6PC*	*G6PC3*	*G6PD*	*GATA2*
GFI1	*GINS1*	*GTF2E2*	*GTF2H5*	*GUCY2C*	*HAX1*	*HELLS*	*HMOX1*	*HPS1*	*HPS3*
HPS4	*HPS5*	*HPS6*	*HTRA2*	*HYOU1*	*ICOS*	*ICOSLG*	*IFIH1*	*IFNAR1*	*IFNAR2*
IFNGR1	*IFNGR2*	*IGLL1*	*IKBKB*	*IL10*	*IL10RA*	*IL10RB*	*IL12B*	*IL12RB1*	*IL12RB2*
IL17F	*IL17RA*	*IL17RC*	*IL1RN*	*IL21*	*IL21R*	*IL23R*	*IL2RA*	*IL2RB*	*IL2RG*
IL36RN	*IL6R*	*IL6ST*	*IL7R*	*IRAK4*	*IRF2BP2*	*IRF4*	*IRF7*	*IRF8*	*IRF9*
ISG15	*ITCH*	*ITGAM*	*ITGB2*	*ITK*	*JAGN1*	*JAK1*	*JAK3*	*KDM6A*	*KMT2A*
KMT2D	*LAMTOR2*	*LAT*	*LCK*	*LCT*	*LIG1*	*LIG4*	*LIPA*	*LPIN2*	*LRBA*
LRRC8A	*LYN*	*LYST*	*MAGT1*	*MALT1*	*MAP3K14*	*MCM4*	*MEFV*	*MKL1*	*MOGS*
MPLKIP	*MS4A1*	*MSN*	*MTHFD1*	*MVK*	*MYD88*	*MYO5B*	*MYSM1*	*NBAS*	*NBN*
NCF2	*NCF4*	*NCSTN*	*NEUROG3*	*NFAT5*	*NFE2L2*	*NFKB1*	*NFKB2*	*NFKBIA*	*NHEJ1*
NHP2	*NLRC4*	*NLRP1*	*NLRP12*	*NLRP3*	*NOD2*	*NOP10*	*NSMCE3*	*OAS1*	*ORAI1*

OSTM1	*OTULIN*	*PARN*	*PAX1*	*PEPD*	*PGM3*	*PIK3CD*	*PIK3R1*	*PLCG2*	*PMM2*
PNP	*POLA1*	*POLD1*	*POLE*	*POLE2*	*POLR3A*	*POMP*	*PRF1*	*PRKCD*	*PRKDC*
PSENEN	*PSMA3*	*PSMB4*	*PSMB8*	*PSMG2*	*PSTPIP1*	*PTPRC*	*RAB27A*	*RAC2*	*RAG1*
RAG2	*RANBP2*	*RASGRP1*	*RBCK1*	*RELA*	*RELB*	*RFX5*	*RFXANK*	*RFXAP*	*RHOH*
RIPK1	*RMRP*	*RNASEH2A*	*RNASEH2B*	*RNASEH2C*	*RNF113A*	*RNF168*	*RNF31*	*RNU4ATAC*	*RORC*
RPSA	*RTEL1*	*SAMD9*	*SAMD9L*	*SAMHD1*	*SAR1B*	*SCO2*	*SEC61A1*	*SEMA3E*	*SERPING1*
SH2D1A	*SH3BP2*	*SH3KBP1*	*SI*	*SIAE*	*SKIV2L*	*SLC26A3*	*SLC29A3*	*SLC35C1*	*SLC37A4*
SLC39A7	*SLC46A1*	*SLC5A1*	*SLC7A7*	*SLC9A3*	*SLX4*	*SMARCAL1*	*SMARCD2*	*SNX10*	*SP110*
SPINK5	*SPINT2*	*SPPL2A*	*SRP54*	*SRP72*	*STAT1*	*STAT2*	*STAT3*	*STAT4*	*STAT5B*
STIM1	*STK4*	*STN1*	*STX11*	*STX3*	*STXBP2*	*TAOK2*	*TAP1*	*TAP2*	*TAPBP*
TAZ	*TBX1*	*TCF3*	*TCIRG1*	*TCN2*	*TERC*	*TERT*	*TFRC*	*TGFB1*	*TGFBR1*
TGFBR2	*THBD*	*TICAM1*	*TIMM50*	*TINF2*	*TLR3*	*TMC6*	*TMC8*	*TMEM173*	*TNFAIP3*
TNFRSF11A	*TNFRSF13B*	*TNFRSF13C*	*TNFRSF1A*	*TNFRSF4*	*TNFRSF6B*	*TNFRSF9*	*TNFSF11*	*TNFSF12*	*TONSL*
TOP2B	*TP63*	*TPP2*	*TRAF3*	*TRAF3IP2*	*TREX1*	*TRNT1*	*TTC37*	*TTC7A*	*TYK2*
UNC13D	*UNC45A*	*UNC93B1*	*UNG*	*USB1*	*VAV1*	*VPS13B*	*VPS45*	*WAS*	*WDR1*
WIPF1	*WRAP53*	*XIAP*	*ZAP70*	*ZBTB24*	*ZCCHC8*	*ZNF341*			

Table 5: Invitae primary immunodeficiency genes panel.

3.4 Functional assay

Pathogenicity of mutations was established with appropriate functional assay, when available, above all to clarify the significance of VUS which are explained in Supplementary materials.

3.5 Transcriptome analysis

Transcriptomic analysis was performed on peripheral whole blood cells of the patient by RNA sequencing.

Differential gene expression pipeline was run in comparison to a group of young healthy controls.

Principal component analysis (PCA), useful for data visualization, was conducted with DESeq2 (v. 1.32.0) (56), to define and display the overall variability between samples considering the 5000 most variable genes detected by the specific function intrinsic to this R package.

The most representative genes were selected by fold change greater than 2-fold increase/decrease and adjusted p-value <0.05. According to the resulting pipeline, the pathway enrichment analysis was carried out by Functional Annotation Tool, DAVID Bioinformatics Resources 6.8, NIAID/NIH, to investigate possible overexpressed biological processes considering an adjusted p-value <0.01.

4. RESULTS & DISCUSSION

4.1 Patients recruitment

In these 3 years we enrolled 30 patients, 15 complying criteria A (autoimmune phenotype), 12 complying criteria B (allergic phenotype) and 3 overlapping both criteria (fig. 9).

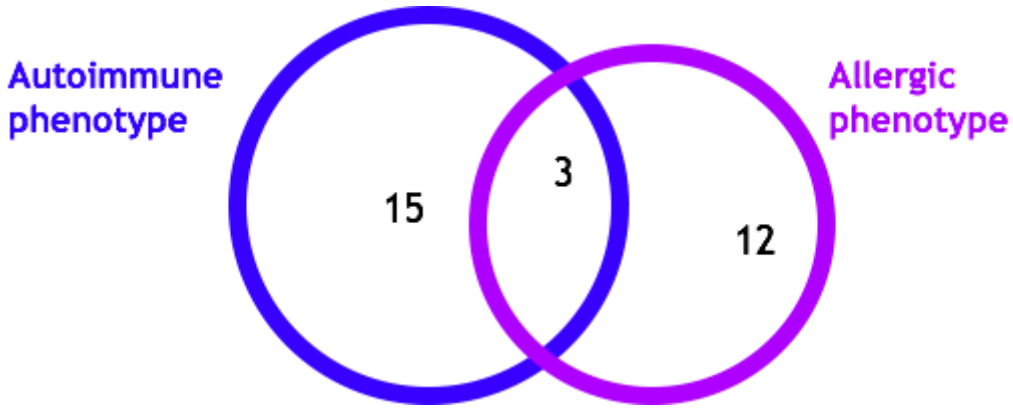


Fig. 9: enrolled patients divided in the two recruitment criteria.

Clinical data of our patients were collected in a database and the group of cases according to criteria A show a picture of multiple autoimmunities (fig. 10A) unlike those that reflect criteria B in which it is observed a prevalence of cutaneous defects (fig. 10B), while there are 4 patients where the two phenotypes described in the inclusion criteria cross each other (fig. 10C).

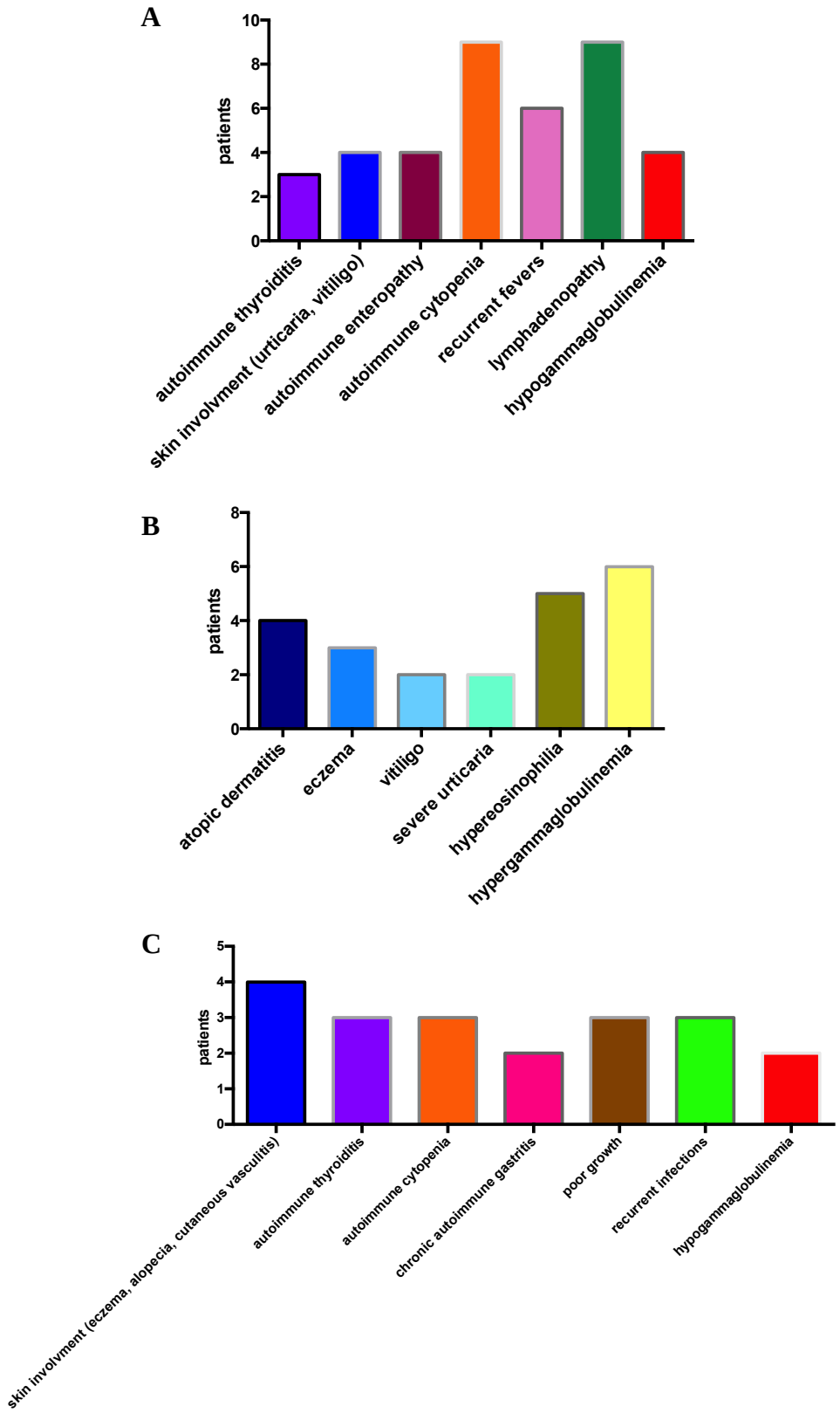


Fig. 10: main clinical aspects of patients enrolled according to criteria A (A), criteria B (B) or according to the intersection between the two criteria (C).

4.2 Genetic positive UDDs cases

For many of these subjects we analyzed the immunophenotype in flow cytometry and made genetic investigations, along with functional assays and transcriptome studies.

According to this combined analysis strategy, 7 patients had a genetic diagnosis, more precisely 3 patients with autoimmune phenotype, 1 patient with allergic phenotype and 3 patients with a phenotype overlapping both of the above. The discovery of these mutations influenced the choice of targeted therapy for some of these patients.

Case 1: RF10. In the first year we enrolled a 10-years-old child (now he is 13 years-old), who fulfilled the inclusion criteria A seeing as he presented early onset autoimmune gastritis with hemorrhagic events with dense lymphocytic infiltrates in the lamina propria of the stomach and the duodenum in addiction to splenomegaly since the age of 2 years together with multiple lymphadenopathy, hypogammaglobulinemia and anemia (probably due to hemorrhagic events).

Gastric biopsies showed features of autoimmune gastritis with lymphocytic inflammation and apoptotic bodies, Anti-gastric wall antibodies were positive (fig.11).

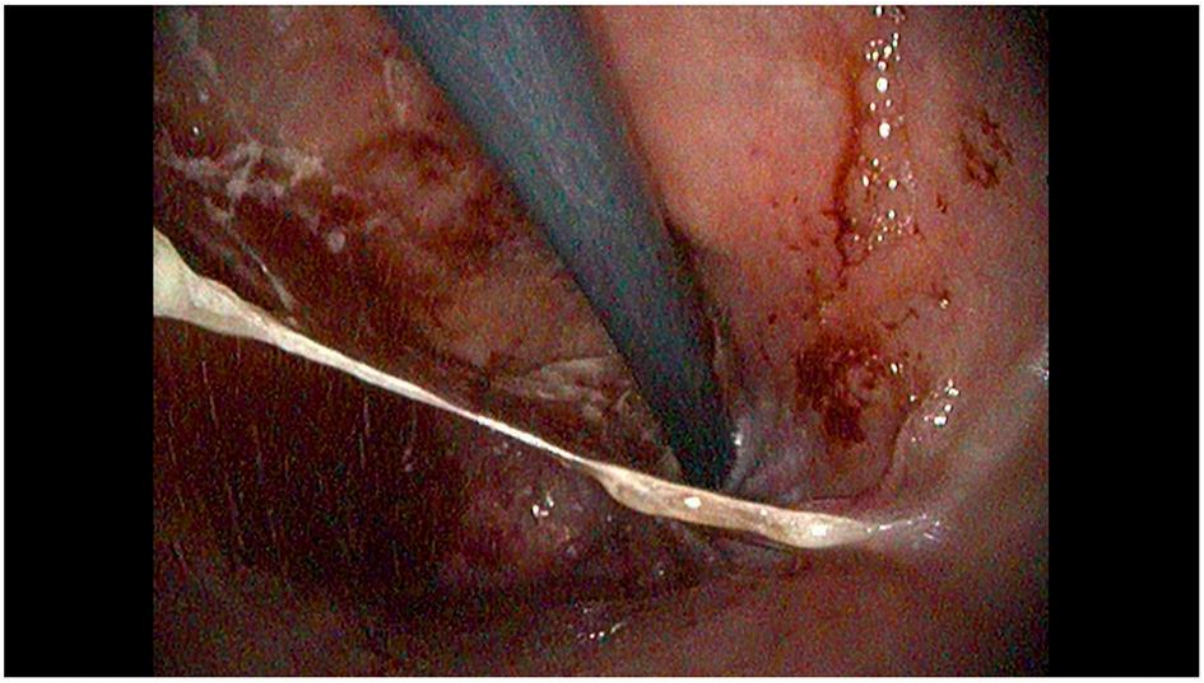


Fig.11: retroverted view of the RF10 proximal stomach, showing diffuse mucosal congestion and hyperemia with discrete amounts of mucus and bleeding.

Laboratory investigation showed microcytic anemia (Hb 8.7 g/dL, MCV 68 fL) with low ferritin (4.3 μg/L) and low vitamin B12 levels (178 pg/mL) and an increased $CD4^-$ $CD8^-$ T

lymphocytes with αβ T cell receptor (double negative T cells, DNT), supporting a possible diagnosis of autoimmune lymphoproliferative syndrome (ALPS), but no mutation was detected in the *FAS* or *FASLG* gene.

Given the complexity of the symptoms, we decided to analyze a TGPS of 10 genes (*CTLA4*, *IL21*, *IL21R*, *LRBA*, *NFNB2*, *PIK3CD*, *PIK3R1*, *RAG1*, *TNFRSF13B*, *TNFRSF13C*) responsible for common variable immunodeficiencies (CVID) using NGS on Ion Torrent platform. We found 2 compound heterozygous mutations in *LRBA* gene (chr 4, NM_006726 → NP_006717), both causing a nucleotide change and an early stop codon (c.C6415T p.R2139X in exon 42 and c.C7315T p.R2439X in exon 49), respectively in PH and BEACH domains, allowing their classifications as pathogenic variants (fig.12) (57).

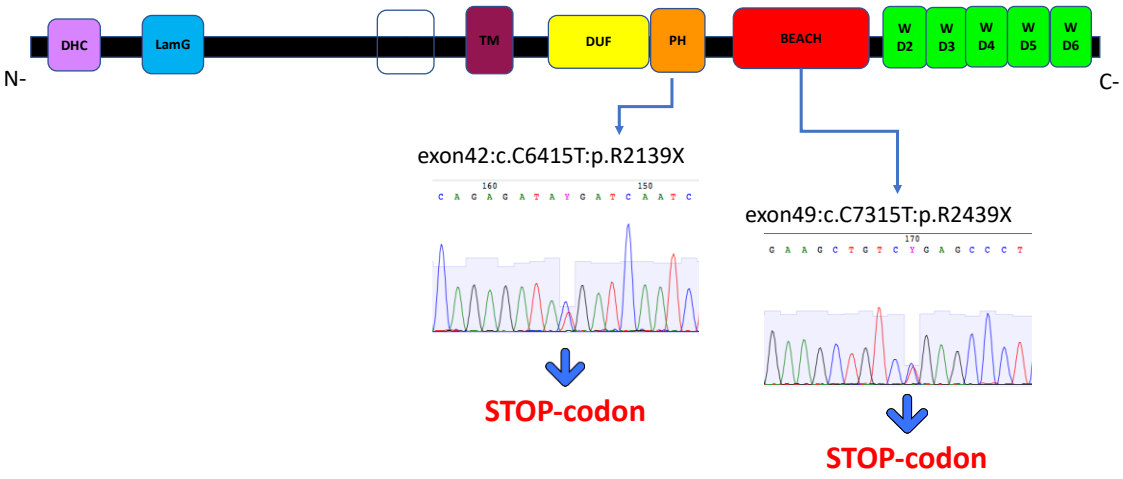


Fig.12: schematic structure of LRBA with the localization of 2 compound heterozygous mutations found in RF10 and their respective electropherograms.

According to the literature, nonsense mutations in these domains lead to the synthesis of a truncated protein which is then degraded or to the synthesis of unstable mRNA (58). Furthermore, we analyzed this gene also in parents and we observed that he inherited a mutation from his father and one from his mother (fig.13).

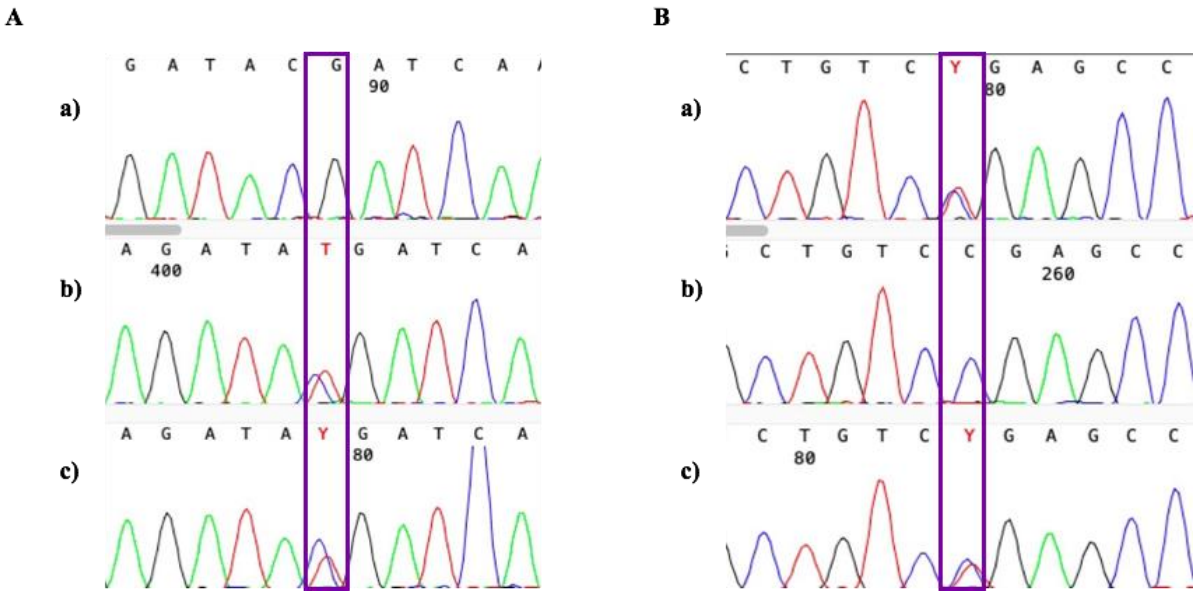
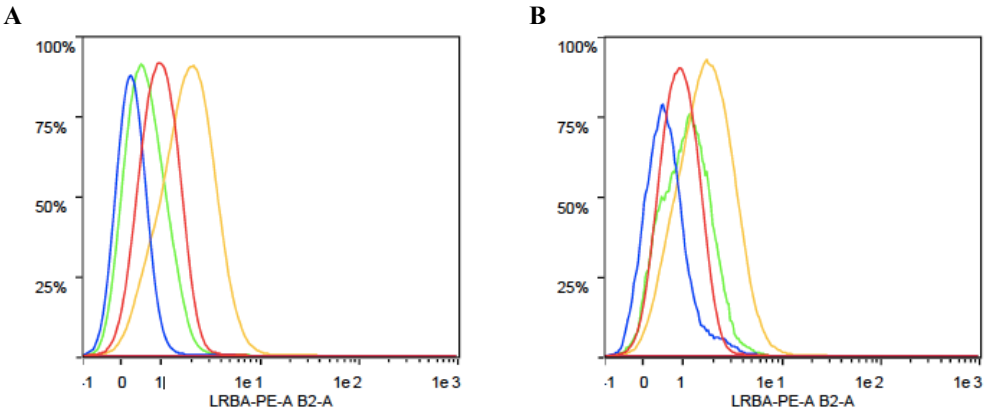


Fig.13: electropherograms resulting from the Sanger sequencing analysis to confirm variant inherited from the mother (A) and that one inherited from the father (B) in *LRBA* gene (a) father's electropherogram (a), mother's electropherogram (b), RF10 proband's (c) electropherogram).

LPS responsive beige–like anchor protein (LRBA) deficiency is an autosomal recessive primary immunodeficiency disease associated with immune dysregulation (59, 60), characterized by a wide range of symptoms, including humoral immune deficiency, lymphoproliferation, hematologic and organ autoimmunity (61), and shares clinical features with inflammatory bowel disease, common variable immunodeficiency, and autoimmune lymphoproliferative syndrome (62). LRBA is a cytoplasmic protein involved in the regulation of intracellular vesicle trafficking especially for the recycling of cytotoxic T-lymphocyte associated protein 4 (CTLA4) on the membrane of regulatory T cells, so in cases of LRBA deficiency we can observed a reduced of membrane expression of CTLA4 in those patients.
Indeed, we analyzed in flow cytometry both intracellular expression of LRBA and surface expression of CTLA4 in PBMCs of our patients compared to a healthy control. As shown in figure 14, both in $CD3^+$ lymphocytes and in activated T lymphocytes ($CD3^+CD69^+$), before and after stimulation with PHA, the LRBA intracellular expression was reduced in the patient compared to the control.

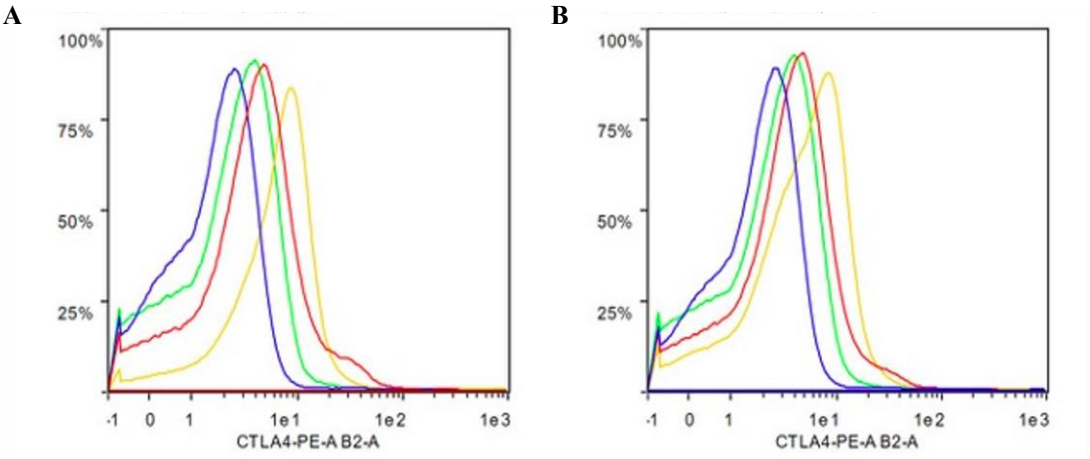


	Sample	MFI
	CD3+_ctrl_nt_LRBA	0,60
	CD3+_ ctrl_PHA_LRBA	1,95
	CD3+_ RF10_nt _ LRBA	0,27
	CD3+_ RF10_ PHA_LRBA	0,98

	Sample	MFI
	CD3+CD69+_ctrl_nt_LRBA	1,13
	CD3+CD69+_ ctrl_PHA_LRBA	1,77
	CD3+CD69+_ RF10_nt _ LRBA	0,55
	CD3+CD69+_ RF10_ PHA_LRBA	0,94

Fig.14: LRBA intracellular expression in RF10 $CD3^+$ lymphocytes population (A) and in activated $CD3^+CD69^+$ T lymphocytes population (B) analyzed in flow cytometry.

Moreover, the cytometric evaluation of CTLA4 surface expression showed CTLA4 expression reduction on peripheral lymphocytes in our patient rather than in healthy control (fig.15).



	Sample	MFI
	CD4+_ctrl_nt_CTLA4	2,36
	CD4+_ ctrl_PMA-ionomycin_CTLA4	7,19
	CD4+_ RF10_nt _ CTLA4	1,76
	CD4+_ RF10 _ PMA-ionomycin_CTLA4	3,80

	Sample	MFI
	lymphocytes_ctrl_nt_CTLA4	2,52
	lymphocytes_ ctrl_PMA-ionomycin_CTLA4	5,32
	lymphocytes_ RF10_nt _ CTLA4	1,97
	lymphocytes_ RF10 _ PMA-ionomycin_CTLA4	3,59

Fig.15: CTLA4 expression on the membrane of RF10 $CD4^+$ T lymphocytes population (A) and in lymphocytes population (B) analyzed before and after stimulation with PMA-ionomycin in flow cytometry.

Furthermore, gene expression analysis by RNA sequencing showed a downregulation of LRBA in our patient compared to a group of healthy controls. Reduced expression of LRBA was confirmed in real-time PCR using the same RNA aliquots from peripheral blood analyzed with RNAseq (table 6).

Gene	**Log2FoldChange**	***p* adjust**
LRBA	-2,00	0,013

Table 6: *LRBA* expression according to RNAseq analysis.

Based on this diagnosis, the boy started off-label treatment with Abatacept, a fusion protein of extracellular domain of CTLA4 and Fc portion of human IgG1, which can be considered a precision therapy both for CTLA4 and for LRBA deficiency, because Abatacept surrogates, at least in part, the defective CTLA4 function (43, 62, 63) (fig.16).

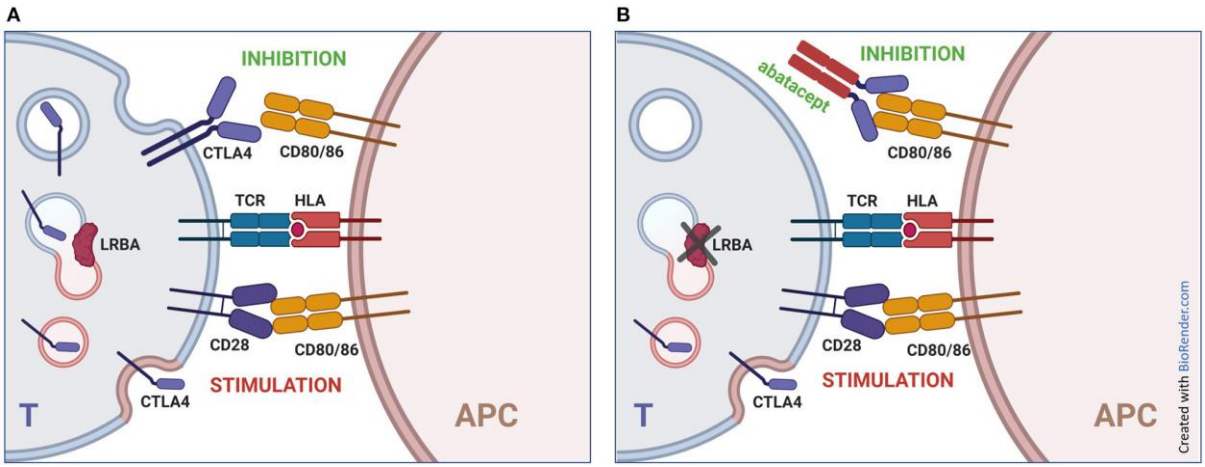


Fig.16: mechanism of action of abatacept in LRBA deficiency. (A) normal immune responses are based on a balance between stimulatory signals (interaction of CD28 on T lymphocytes with CD80 and CD86 on antigen presenting cells) and inhibitory signals (block of CD80 and CD86 by CTLA4 expressed by lymphocytes). (B) biallelic mutations in LRBA result in impaired recycling of CTLA4 to the membrane, leading to reduced expression of the protein and increased lymphocyte stimulation. The addition of abatacept (CTLA4-Ig) partially compensates for the lack of cell-bound CTLA4 (62).

Within a few weeks, the boy had no more nausea or vomiting and after 6 months of treatment, anemia resolved and DNT cells decreased and also endoscopy showed a gastric mucosa without erosive lesions (fig.17).

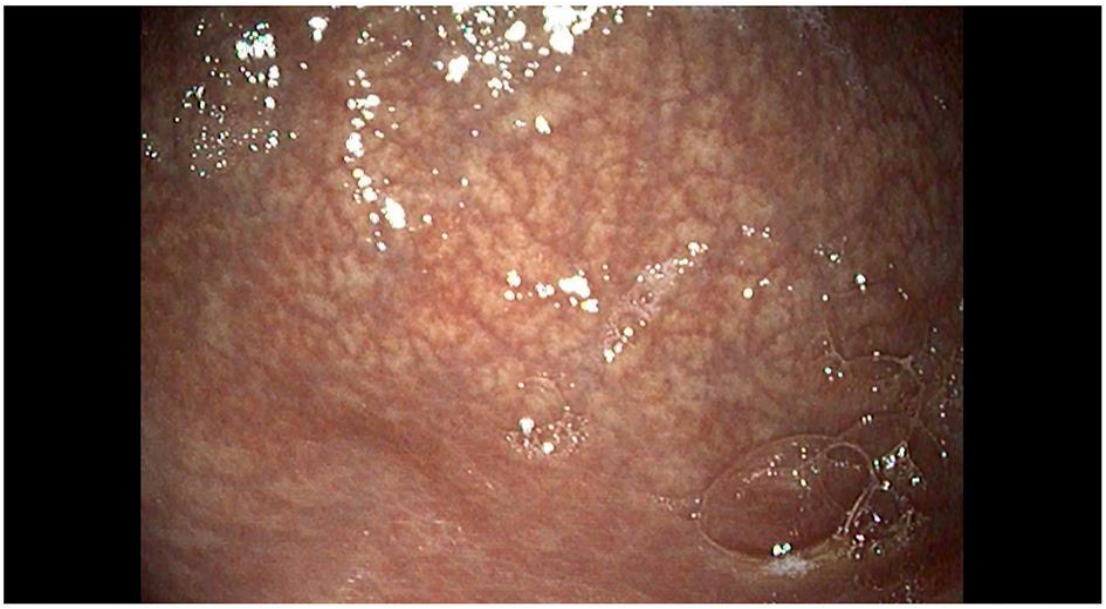


Fig.17: endoscopic view of RF10 large curvature of the stomach showing a normal mucosa with decreased gastric folds.

Case 2: RF28. A lady of 30 years old (now 32 years old) was enrolled according to her autoimmune phenotype which showed an autoimmune thrombocytopenia and severe hypogammaglobulinemia (IgG, IgA and IgM deficiency) since the age of 13. Throughout her medical history, she developed chronic enteropathy with severe episodes of severe gastroenteritis and insulin-dependent type 1 diabetes mellitus (T1DM) and autoimmune thyroiditis in addition to recurrent infections mostly respiratory ones including pneumonias and chronic sinusitis. Flow cytometry analysis of lymphocyte subpopulation pointed out a very low level of $CD19^+$ B lymphocytes with an imbalance of switched memory B lymphocytes and a substantial decrease of recent thymic emigrants T lymphocytes and DNT population in normal ranges. This picture of immune-deficiency with immune-dysregulation and hypogammaglobulinemia led to the analysis of the same panel of genes of case 1. We found a heterozygous mutation (c.C223T p.R75W in exon 2) in *CTLA4* gene (chr 2; NM_005214 → NP_005205), confirmed in Sanger Sequencing (fig.18), which was described as causative of the disease phenotype by HGMD professional database, more precisely this mutation is responsible for the onset of CTLA4 haploinsufficiency with autosomal dominant inheritance.

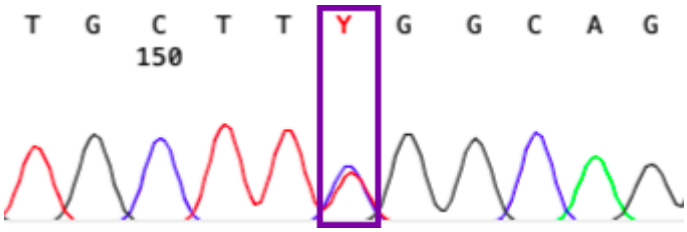
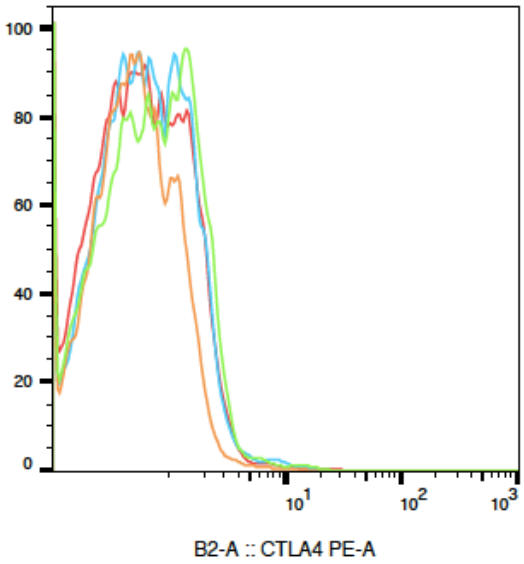


Fig.18: electropherograms resulting from the Sanger sequencing analysis to confirm variant in *CTLA4* gene of RF28.

CTLA4 is a negative regulator of T cell activation working as an antagonist of the co-stimulatory molecule CD28, which binds its receptor CD80/CD86 on antigen presenting cells (APC) providing the so-called second signal for T cell activation. CTLA4 interrupts the immune response competing with CD28 for binding to the same receptor (29305966).
Literature data report superimposable clinical and laboratory evidences to the phenotype of our patient (29305966), but in flow cytometry analysis we did not see any particular differences on the CTLA4 surface expression of $CD4^+$ T cells, between patient and healthy control before and after treatment with PMA-ionomycin (fig.19).



	Sample	MFI
	CD4+_ctrl_nt_CTLA4	0,48
	CD4+_ ctrl_PMA-ionomycin_CTLA4	0,58
	CD4+_ RF28_nt _ CTLA4	0,41
	CD4+_ RF28 _ PMA-ionomycin_CTLA4	0,65

Fig.19: CTLA4 expression on the membrane of RF28 T lymphocytes $CD4^+$ population analyzed before and after stimulation with PMA-ionomycin in flow cytometry.

Patient started treatment with Abatacept just one year ago and the enteropathy seems to be stationary and no worsening was reported. However, lung disease showed a slow worsening that was not contrasted by the medication.

Case 3: RF25. Thanks to the collaboration with an external service, Invitae, in San Francisco (USA), we found a heterozygous mutation (c.G160A p.A54T in exon 2) in *CTLA4* causing missense changes on protein structure and function in a 37 years old lady. SIFT, PolyPhen-2 and Align-GVGD database suggest that this variant is likely to be disruptive and related to CTLA4 haploinsufficiency that can explain the clinical history of the lady characterized by recurrent fevers, thrombocytopenia, autoimmune hemolytic anemia, leukopenia, recurrent infections and severe hypogammaglobulinemia (inclusion criteria A). Moreover, lymphocyte subpopulation investigation underlined an almost total absence of B lymphocytes and a very low percentage of RTE and DNT.
Variant was confirmed in Sanger and we also analyzed the segregation of the variant in her parents, brother and daughter and the same variant is present in mother and brother (fig. 20).

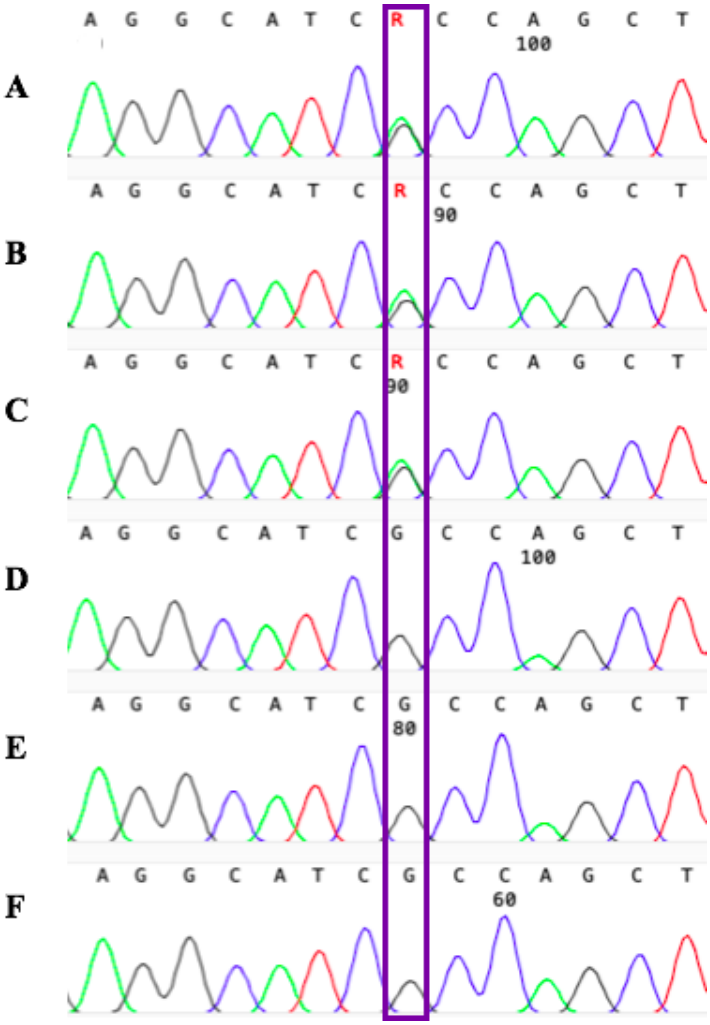
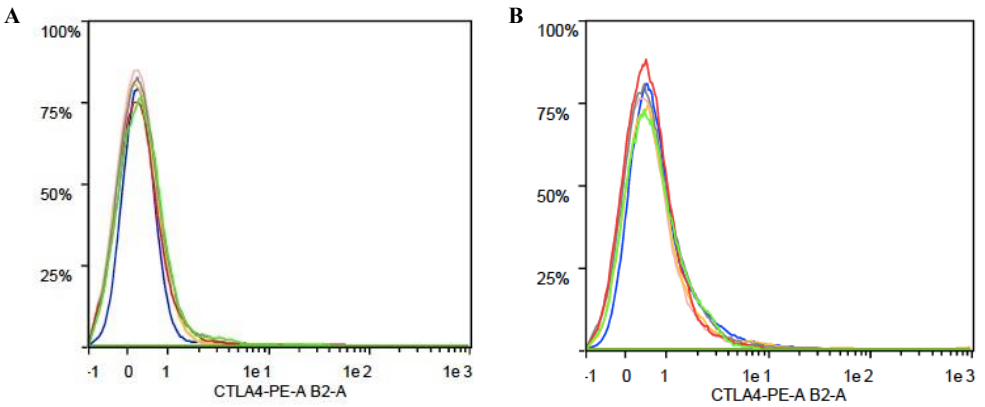


Fig.20: electropherograms of segregation analysis to confirm the variant in *CTLA4* by Sanger sequencing: we found the same mutation of RF25 proband (A), in her mother (B) and her brother (C). Her father (D), her daughter (E) and her maternal aunt (F) were wild-type.

As in the previous case of variant in CTLA4, we analyzed the surface expression of the protein in $CD4^+$ T cells population but we did not see any differences between healthy control and between subjects with mutation or not also in this case and with a different protocol of PBMCs stimulation (fig.21).



	Sample	MFI
	CD3+ CD4+_ctrl_ns_CTLA4	0,30
	CD3+ CD4+_ RF25_ns _CTLA4	0,25
	CD3+ CD4+_ RF25 daughter_ns _CTLA4	0,25
	CD3+ CD4+_ RF25 father_ns _CTLA4	0,29
	CD3+ CD4+_ RF25 brother_ns _CTLA4	0,26
	CD3+ CD4+_ RF25 mother_ns _CTLA4	0,32

	Sample	MFI
	CD3+ CD4+_ctrl_PHA_CTLA4	0,66
	CD3+ CD4+_ RF25_PHA _CTLA4	0,49
	CD3+ CD4+_ RF25 daughter_PHA _CTLA4	0,57
	CD3+ CD4+_ RF25 father_PHA _CTLA4	0,56
	CD3+ CD4+_ RF25 brother_PHA_CTLA4	0,53
	CD3+ CD4+_ RF25 mother_PHA_CTLA4	0,60

Fig.21: Fig: CTLA4 expression on the membrane of RF25 $CD3^{+}CD4^{+}$ T lymphocytes population before stimulation with PHA (A) and in $CD3^{+}CD4^{+}$ population after stimulation (B) analyzed in flow cytometry.

Moreover, the enrichment pathways analysis, obtained by the study of the differential expressed genes of this patient compared to the group of healthy controls, displayed an over-representation of signaling related to B cells population. It is not clear the biological significance of these results, a probable explanation may be linked to the almost total absence of this cell subset or to the IgG replacement therapy to control the severe hypogammaglobulinemia (fig.22)

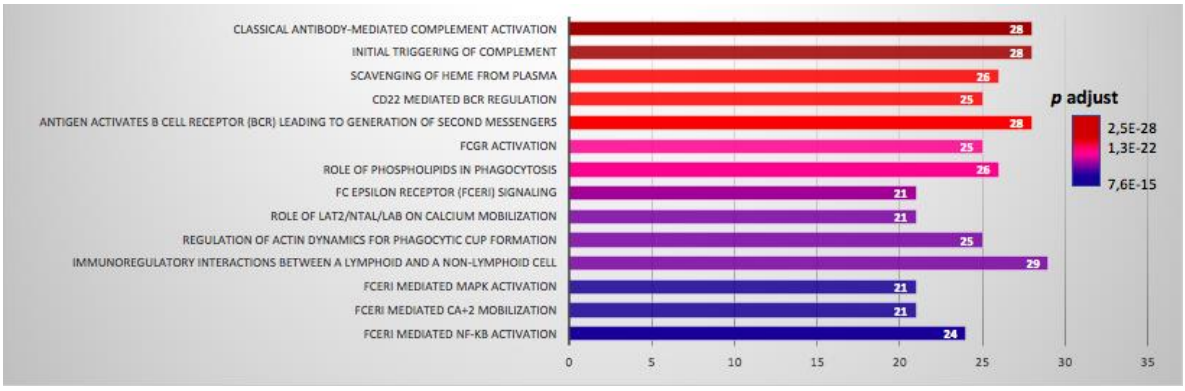


Fig.22: RF25 fourteen most enriched pathways that are ordered by p-values displayed by gradient color scale that represents the full range of p-values (lowest p-values in red) according to REACTOME_PATHWAY database, the analysis has been processed by Functional Annotation Tool, DAVID Bioinformatics Resources 6.8, NIAID/NIH.

Case 4: RF11 The first enrolled patient who overlapped both inclusion criteria was a 34 years old lady (died this year at the age of 37) with early onset of multiple autoimmune features such as primary hypothyroidism and autoimmune enteropathy which led to problems of caloric-protein malnutrition bringing her into parenteral nutrition. In adulthood, she developed rheumatoid arthritis, mild brain atrophy with calcifications, and nephrotic syndrome. Furthermore, she developed a trilinear autoimmune cytopenia (haemolytic anemia, thrombocytopenia, neutropenia) that got worse and worse. To close the clinical picture we must mention recurrent viral infections, especially herpes zoster, and bacterial ones which were difficult to treat due to multiple allergies to antibiotics.

Flow cytometry immunophenotyping yielded normal results, but hypergammaglobulinemia of IgG together with hypogammaglobulinemia has been noticed.

According to the complexity of this set of symptoms WES analysis was made by Biodiversa, an external service, and we found a heterozygous mutation (c.735ofG p.A247Qfs*46 in exon 4) in the extracellular domain of protein encoding by *FASLG* gene (NM_000639.3 → NP_000630.1), which caused a reading frameshift linking to the creation of a new STOP codon 46 amino acids downstream of the deletion eventually generating a longer protein (293 aa) rather than the endogenous one (281 aa). This variant was confirmed in Sanger sequencing and the segregation analysis showed a wild-type sequence both in her mother and her sister (DNA of her father was not available) (fig.23).

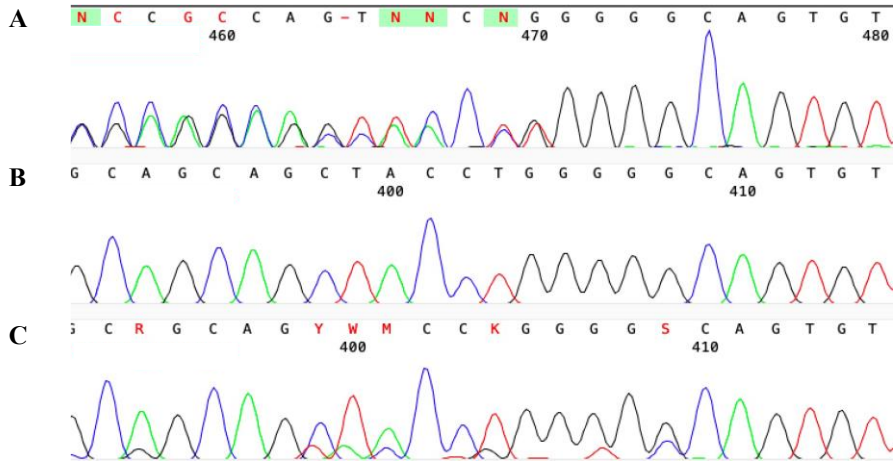


Fig.23: electropherogram resulting from the Sanger sequencing analysis to confirm variant in *FASLG* gene in RF11 (A) and electropherograms of her sister (B) and her mother (C) for the segregation analysis.

FASLG (FAS ligand) has an essential role in the apoptosis induction thanks to its binding with FAS and this pathway is also essential in the immune system for cellular death induced by T cells. Defects on this gene can be correlated with ALPS (autoimmune lymphoproliferative syndrome) characterized by chronic lymphoproliferation (fig.24).

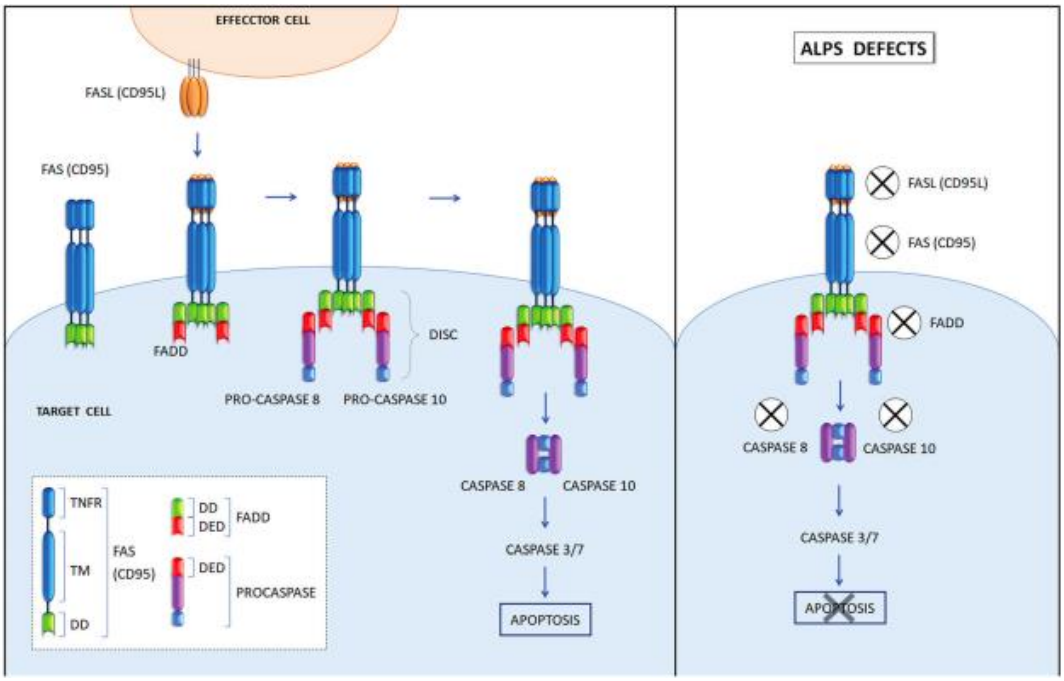


Fig.24: schematic view of FAS/FASLG pathway and ALPS defects (64).

Although there are no specific functional studies, the variant, by its nature, is to be considered probably pathogenic (likely pathogenic), according to the ACMG/AMP guidelines (65).
However, the evaluation of enrichment pathways has highlighted an imbalance in mechanisms of lymphoproliferation which was more accentuated in the second analysis of RNAseq than in the first one. The RNA extracted for the second RNAseq was obtained in the acute phase of the disease, when the patient's condition began to worsen, therefore we can probably attribute the variance between the two pathway distributions to the difference in clinical pictures afferent to the two samples for RNA (fig.25).

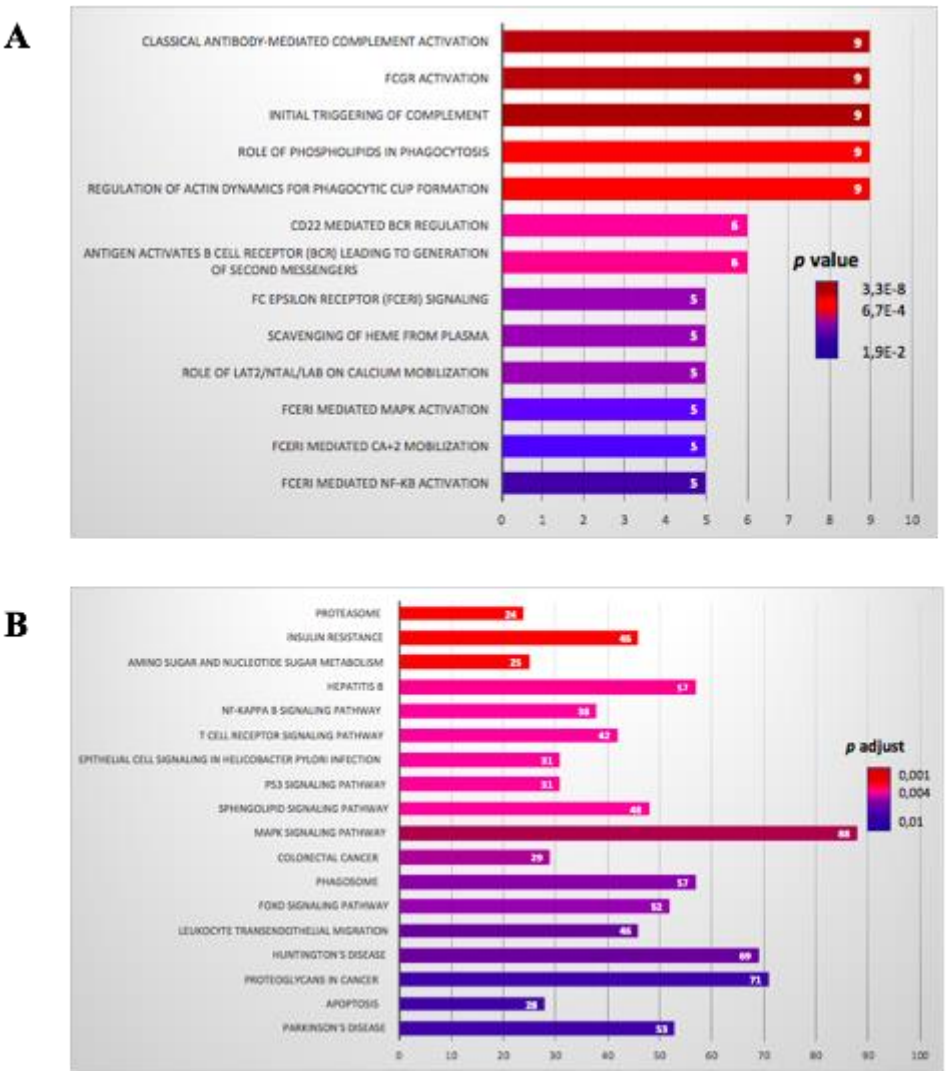


Fig.25: enrichment pathways from the first blood sample of RF11 for RNA extraction (A) and from the second one (B) according to REACTOME_PATHWAY database, the analysis has been processed by Functional Annotation Tool, DAVID Bioinformatics Resources 6.8, NIAID/NIH.

Moreover, table 7 displays the expression levels of *FASLG* and *LRBA* genes, *FAS* gene and other ones involved in apoptosis, taken from the second RNAseq analysis compared to a group of healthy controls to confirm the dysregulation of lymphoproliferation. The low expression of *FASLG* and *LRBA* genes was also confirmed by real-time PCR using the same RNA aliquots employed for RNAseq.

Gene	Log2FoldChange	*p* adjust
FASLG	-8,89	0,00028
FAS	-1,60	0,0030
LRBA	-3,75	3,39E-10
BAG2	-3,20	1,18E-6
BCL2	-2,00	0,0014
BAX	4,34	5,59E-23

Table 7: expression of a selection of genes involved in the regulation of cell proliferation.

The discovery of FASLG mutation did not lead to the choice of a targeted therapy for our patient who was in treatment with cyclosporine, cortisone, Tacrolimus, Gabapentin for convulsions and Abatacept that has improved her arthritis. This improvement due to the use of Abatacept and the result of *LRBA* gene expression obtained by RNAseq led us to wonder if there was a link between FASLG and LRBA. A possible interaction may rely on the ability of both proteins to influence the pathway of mTOR/PI3K. What we know from the literature is that FAS/FASLG pathway inhibits mTOR/PI3K pathway, and in cases of ALPS PI3K pathway is hyperactivated. However, CTLA4 can also regulate PI3K by directly interacting with it (fig.26) (8).

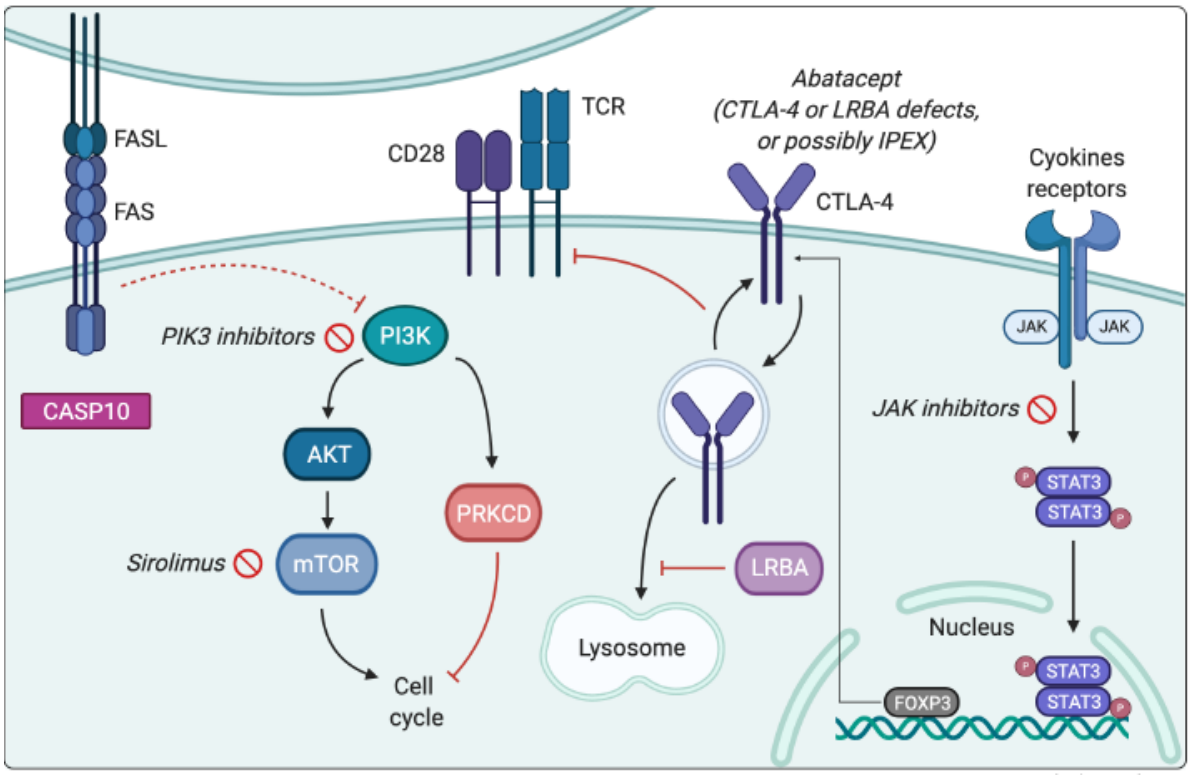
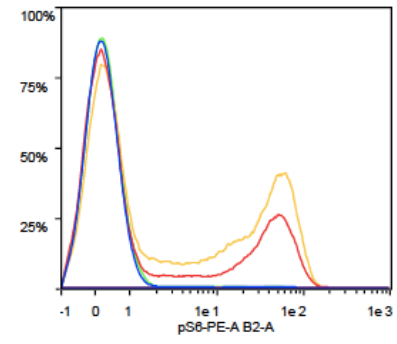


Fig.26: crosstalk between pathway involved in pathologies like CTLA4 and LRBA defects, IPEX and ALPS (8).

Moreover, it is reported that activated LRBA-deficient $CD4^+$ and $CD8^+$ T cell subsets show an impaired mTORC activity with a reduced phosphorylation of S6 (pS6), a downstream mTORC substrate (58, 66).

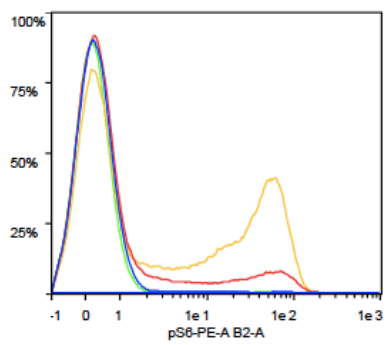
Thus, we have decided to evaluate the phosphorylation levels of S6 in our patient with LRBA deficiency (RF10) in flow cytometry but also in the 2 patients with CTLA4 haploinsufficiency (RF25 and RF28) given the close link between the two proteins and the influence that LRBA may have on the level of function of CTLA4. We stimulated PBMCs of our patients and healthy control with Dynabeads Human T-activator CD3/CD28 and we calculated the phosphorylation levels of S6 in $CD4^+$ T acttivated and we noticed a lower phosphorylation in all our patients than healthy control (fig.27).

A



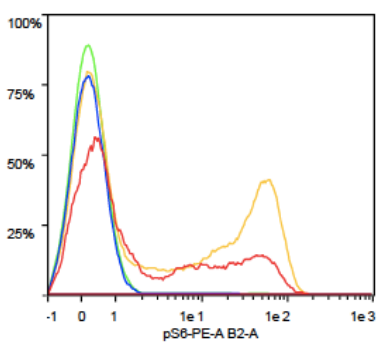
	Sample	MFI	% CD4+ pS6+
	CD4+_ctrl_nt_pS6	0,22	0,13%
	CD4+_ ctrl_beads CD3/CD28_pS6	1,02	45,17%
	CD4+_ RF10_nt _ pS6	0,20	0,19%
	CD4+_ RF10_ beads CD3/CD28_pS6	0,48	30,89%

B



	Sample	MFI	% CD4+ pS6+
	CD4+_ctrl_nt_pS6	0,22	0,13%
	CD4+_ ctrl_beads CD3/CD28_pS6	1,02	45,17%
	CD4+_ RF28_nt _ pS6	0,27	0,45%
	CD4+_ RF10_ beads CD3/CD28_pS6	0,41	15,48%

C



	Sample	MFI	% CD4+ pS6+
	CD4+_ctrl_nt_pS6	0,22	0,13%
	CD4+_ ctrl_beads CD3/CD28_pS6	1,02	45,17%
	CD4+_ RF25_nt _ pS6	0,21	0,41%
	CD4+_ RF25_ beads CD3/CD28_pS6	0,81	34,55%

Fig.27: S6 phosphorylation levels in RF10 (A), RF28 (B) and RF25 (C), before and after stimulation.

Therefore, according to these last results we might say that mTOR/PI3K could be considered in the study of patients with CTLA4 haploinsufficiency and LRBA deficiency analyzing pS6 in PBMCs.

Case 5: RF22 A 24-year-old lady, enrolled according to both inclusion criteria, presented a history of recurrent oral candida infections (about 2 episodes every year) with the recurrent occurrence of aphthous stomatitis (RAS) since the age of 2 years and the development of esophagitis. Moreover, she showed autoimmune features such as autoimmune thyroiditis, haemolytic anemia, autoimmune chronic gastritis and a skin involvement with the appearance of hypopigmented patches. Laboratory investigation found positive anti-ANA antibodies, anti-dsDNA, anti-gastric parietal cells and ESR values always very high in addition to persistent increase in inflammation indices, so we had a situation of systemic inflammation, but the immunophenotype was normal.

At the beginning, the initial diagnosis was Behcet's disease (BD), a systemic inflammatory disease involving primarily the oral and genital mucosa, skin and eyes, since the most common cause of RAS and inflammation is usually BD where aphthosis is often severe with multiple ulcerations (67, 68). However, the onset of BD is more common in young adulthood (25-30 years) and only occasionally in children, so the onset of the disease in the last category should

raise the suspicion of an underlying genetic disorder (67, 68), as in the case of our patient. In agreement, a recent study in a pediatric series of BD showed that a high proportion of patients carried pathogenic mutations in genes associated with monogenic autoinflammatory disorders characterized by the presence of RAS (68, 69).

Therefore, we sequenced a panel of 17 genes listed below: *TNFAIP3* (NM_001270508 → NP_001257437), *STAT1* (NM_007315 → NP_009330), *DNASE2* (NM_ 001375 → NP_001366), *DNASE1L3* (NM_004944 → NP_ 004935), *TMEM173* (NM_198282 → NP_938023), *PRKCD* (NM_006254 → NP_006245), *TREX1* (NM_033629 → NP_ 338599), *SAMHD1* (NM_015474 → NP_056289), *IFIH1* (NM_022168 → NP_071451), *DNASE1* (NM_005223 → NP_005214), *ISG15* (NM_005101 → NP_005092), *PTPN22* (NM_015967 → NP_057051), *CTLA4* (NM_005214 → NP_ 005205), *STAT4* (NM_003151 → NP_003142), *TLR4* (NM_ 138554 → NP_612564), *RORC* (NM_005060 → NP_ 005051), *RC3H1* (NM_001300850 → NP_001287779), which included also genes involved in monogenic disordes previously classified as BD.

An heterozygous missense mutation (c.A1721C_p.N574T in exon 20) in *STAT1* (NM_007315 → NP_009330) was found in our patient and confirmed in Sanger sequencing (fig.28), which can explain her set of symptoms.

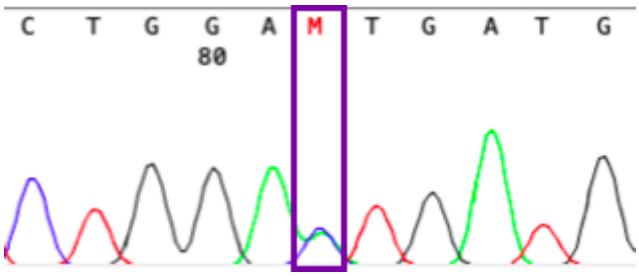
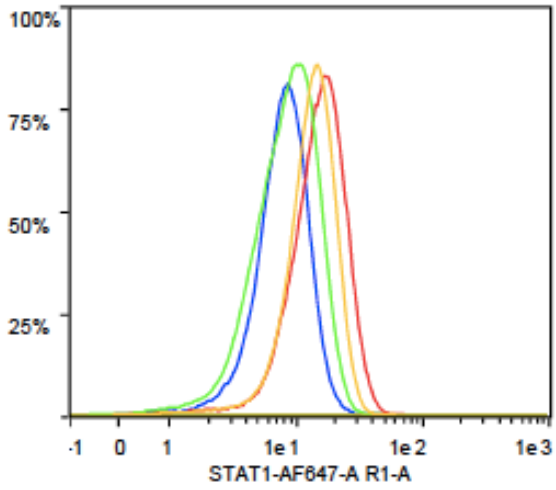


Fig.28: electropherograms resulting from the Sanger sequencing analysis to confirm variant in *STAT1* gene in RF22.

In silico prediction tools SIFT, LRT and Mutation Taster classified this variant as damaging and moreover it was previously described as gain of function mutation of the protein; it is similarly predicted to have a pathogenic effect and causative of STAT1 GOF (gain of function) (70), an autosomal dominant disease, defined as PID with immunodeficiency and immune dysregulation in 2011 (71), This condition was typified by recurrent non-periodic fever with orogenital ulcerations, recurrent chest infections, autoimmune disorders and rheumatologic manifestations, due to increased activation of interferon stimulated genes (45, 70, 72).

Since it was demonstrated that STAT1 GOF patients show an increase of total STAT1 protein levels (72), we evaluated the STAT1 expression in PBMCs of patient compared with expression in PBMCs of healthy control by flow cytometry analysis. As shown in the figure (fig.29), the patient did not display a clear increase of STAT1 expression.



	Sample	MFI
	CD4+_ctrl_nt_STAT1	8,41
	CD4+_ ctrl_IFNα_STAT1	16,61
	CD4+_ RF22_nt _ STAT1	9,23
	CD4+_ RF22_ IFNα_STAT1	14,45

Fig.29: STAT1 intracellular expression in RF22 $CD4^+$ T cells before and after stimulation with IFNα in flow cytometry.

The discovery of the mutation had a positive influence on the reorganization of the therapeutic plan, which included mycophenolate and colchicine that partially controlled the disease and now low dose of JACK-inhibitor, baricitinib, was proposed to improve the control of symptoms.

Case 6: RF30 In the last month of my PhD project a child of 8 years old was enrolled with a picture of diarrhea from 6 months of age resulting in poor growth (reduction of IGF1 but normal levels of growth hormone) and appearance of atopic eczema from the first weeks of life. The laboratory investigations revealed leukocytosis (increase of $CD8^+$ T memory) with increment of platelets number, hypereosinophilia and increase of total IgE (food allergy) with a mild decrease of IgG.

During his life multiple dental agenesis were observed and episodes of diarrhea persisted until the age of 8 years old when the serological analysis to assess the presence of anti-endomysial IgA was positive, so coeliac disease was diagnosed (criteria B).

WES analysis performed with the collaboration of our Institute of Genetics found a hemizygous mutation (c.748_750ofAAG_p.K250del) in *FOXP3* gene which could explain his clinical situations. Mutation in *FOXP3* gene was associated with IPEX syndrome, a monogenic immune disorder characterized by an impaired development of Treg cells, resulting in failure of peripheral immune tolerance, with autoimmunity and allergic manifestations (29). The disease typically arises in infancy with enteropathy, cutaneous disorders with eczema and nail changes or some teeth agenesis as in case of our patient, and endocrinopathies such as thyroiditis together with several other autoimmune manifestations (20, 29, 73), but the clinical spectrum is much more heterogeneous. Consonni et al reported that some IPEX cases are characterized by a less severe early-onset phenotype, such as mild eczema and tolerated diarrhea or early-onset type 1 diabetes mellitus (T1MD) without gastrointestinal involvement and laboratory investigation revealed that immunoglobulin levels may change from normal to reduce while IgE could be in range or elevated and negative autoantibodies (74) (fig.30).

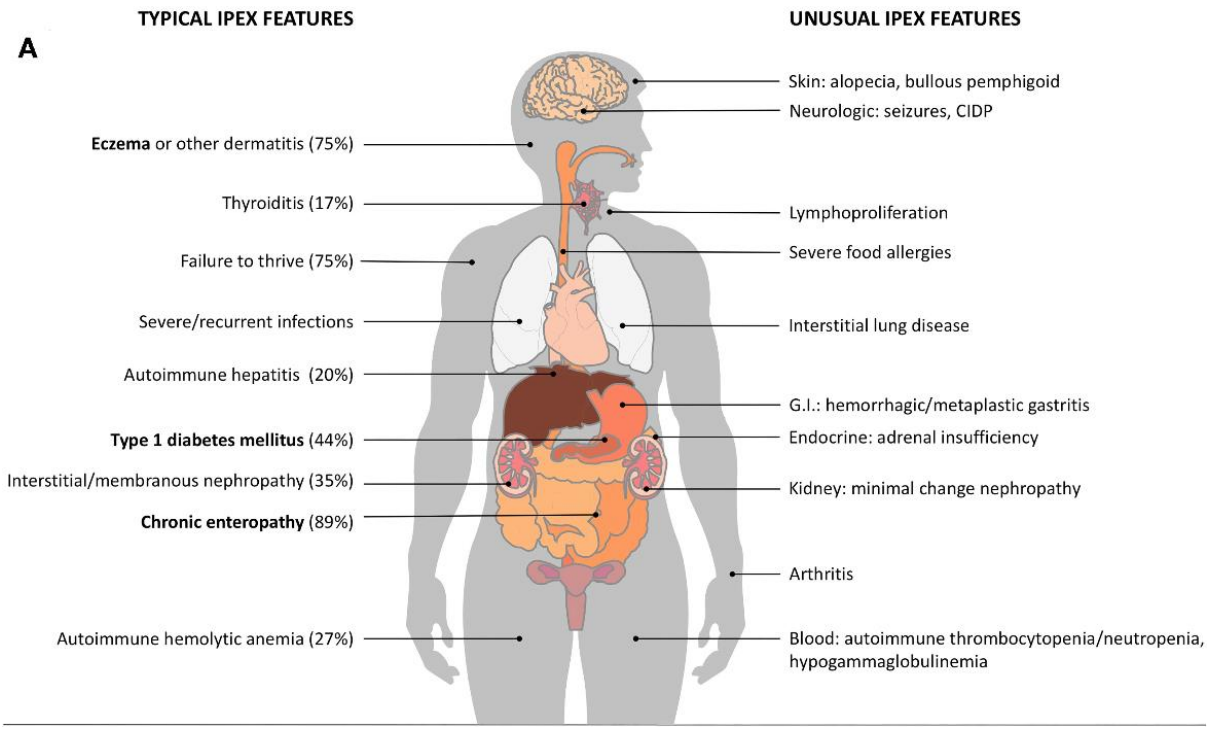


Fig.30: typical and unusual clinical features in IPEX with respective percentages of manifestation (74).

There are several variants of *FOXP3* gene correlated with variable IPEX phenotype including that found in our patient, p.K250del, which is localized to the leucine zipper domain (fig.31), required for FOXP3 homodimerization and transcriptional activity. Therefore, impaired formation of FOXP3 dimerization structure reduces protein expression and destabilizes its expression (74) which could be investigated also in our patient by flow cytometry analysis.

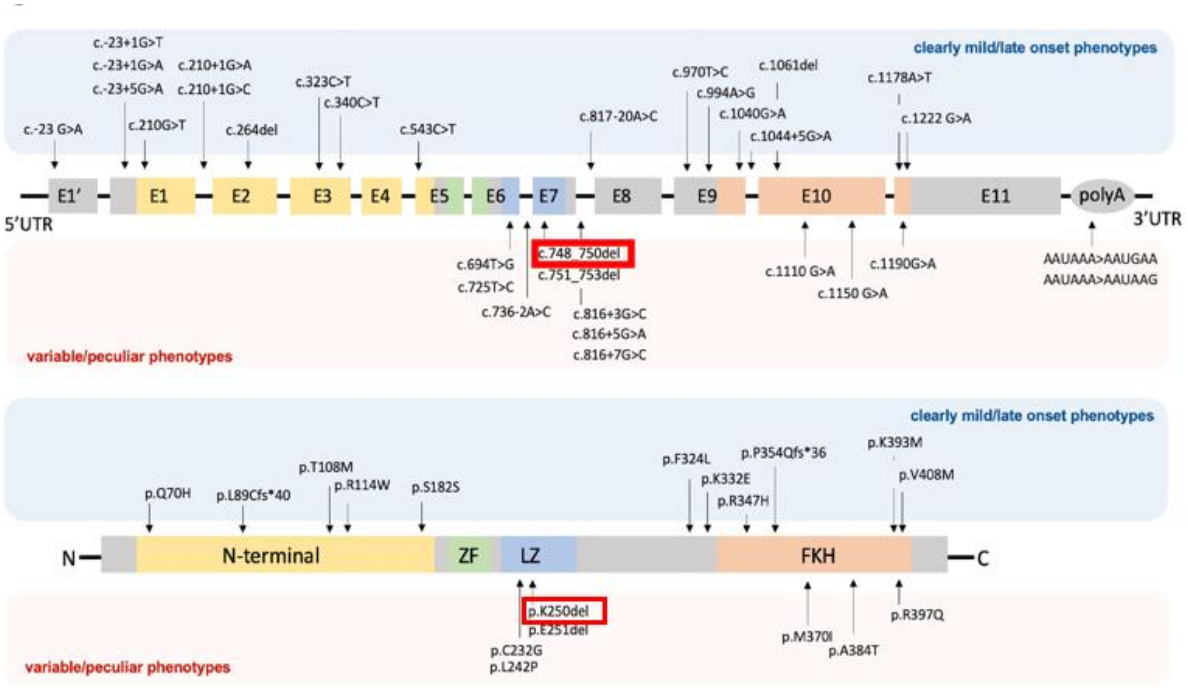


Fig.31: FOXP3 gene and protein structure showing mutations associated with atypical IPEX phenotypes. Mutation of RF30 is framed in red (74).

According to the discovery of mutation, clinicians are considering an update of the treatment plan that now includes betamethasone for the treatment of eczema, sirolimus to preserve residual Treg function (75) and methimazole due to the recent hyperthyroidism report, all accompanied by a gluten-free diet.

Case 7: RF23 The last patient, enrolled in the third year, with a genetic diagnosis is a 43-year-old lady with a combination of autoimmune manifestations such as thyroiditis and gastritis and allergic phenotype including hypogammaglobulinemia in addition to iron deficiency anemia. In her childhood she had episodes of relapsing candida infections with oral aphthosis and the onset of polyarthritis pain and skin involvement from alopecia. Flow cytometry investigation of immunophenotype displayed a strong reduction of switched memory $CD19^{+}$ B cells which could be clarified with her heterozygous missense mutation (c.T310C_p.C104R in exon 3) in *TNFRSF13B* gene (NM_012452.2 → NP_036584.1), coding for the transmembrane activator

and CAML interactor (TACI), a protein belonging to the family of TNF receptors and it is predominantly expressed in peripheral B lymphocytes. Mutation was confirmed by Sanger Sequencing (fig.32).

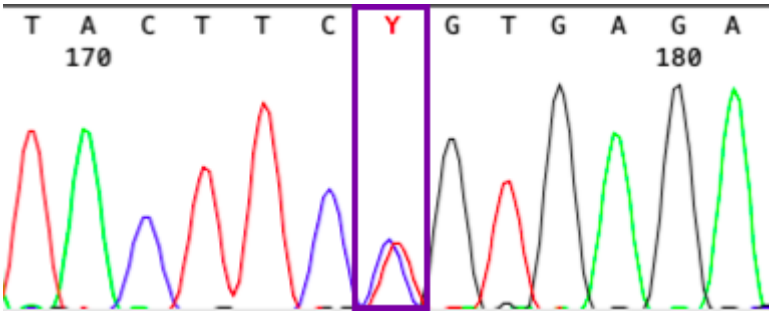


Fig.32: electropherograms resulting from the Sanger sequencing analysis to confirm variant in *TNFRSF13B* gene in RF23.

Mutation was found by INVITAE external service through the clinical exome, a panel of more than 400 target genes involved in PID.

This variant can lead to B-naïve reduction if associated with hypogammaglobulinemia as in this case and it is classified as pathogenetic from HGMD Professional database and literature, associated with common variable immunodeficiency (CVID), even though gnomAD_exome database reports a high frequency for this variant in the control population. Indeed, bioinformatic software InterVar identifies it as "benign", probably due precisely to this high frequency in the control population. However, Freiberger et al demonstrated that this variant is significantly enriched in patients with CVID (76) and also prediction *in silico* software, like CADD phred or Sift, define this variant as probably pathogenetic. So, what we can do in the future is to analyze the segregation of the variant in the family if possible, seeing as it has been noticed that this variant segregates with the disease into multiple families (77, 78)

4.3 Genetic negative UDDs cases

The other 23 enrolled patients did not receive a genetic diagnosis, because we did not find any variants in their WES analysis or clinic exome and the panel of target genes gave a negative result. To investigate the possible genetic variants, we sequenced a specific disease-causing gene when we had a strong clinical suspicion or an immunophenotype supportive of the specific disorder, a panel of target genes when the clinical and immunological phenotype overlapped a group of correlated diseases or WES analysis or clinic exome in remaining cases. For patients with these symptoms the analysis of immunophenotype is usually performed in diagnostic

assistance practices, while the functional assays were guided by clinical data and performed to observe the effects of proteins possibly involved in the pathologies.

In the following table 8 we summarized the clinical and immunophenotype characterization and the genetic and functional assays we made for them.

Patient	**Inclusion Criteria**	**Age of enrollment**	**Clinical Features**	**Immuno phenotype**	**Genetic analysis**	**Functional assays**
RF1	A	55	RF2 father with similar clinical picture	-	nd	nd
RF2	A	20	autoimmune thyroiditis (maternal familiarity); alopecia; maternal familiarity for breast cancer	-	nd	nd
RF3	A	17	trilinear autoimmune cytopenia; urticaria; allergic rhinitis;	RBE (CD10+ CD38+) ↑ CD4/CD8 ↑	WES analysis (Biodiversa)	pSTAT3 ↓
RF4	B	37	severe dermatitis	RBE (CD10+ CD38+) ↑ T regulatory (CD25+) ↓	nd	nd
RF5	B	47	intractable eczema; hypergammaglobulinemia (IgE)	T regulatory (CD25+) ↑ CD4/CD8 ↑	WES analysis (Biodiversa)	CD86 expression

RF6	B	14	urticaria; angioedema; recurrent pericarditis; hypereosinophilia; unexplained fever; paternal familiarity for Hodgkin's lymphoma; hypergammaglobulinemia (IgM)	B lymphocytes CD19+ ↑ T lymphocytes CD8+ ↑ T regulatory (CD25+) ↑ CD4/CD8 ↓ RTE CD4+ lymphocytes ↓	nd	nd
RF7	B	2	severe atopic dermatitis; severe eczema; food allergy; hypereosinophilia; persistent increase in inflammation indices; hypergammaglobulinemia (IgE)	CD4/CD8 ↑ RTE CD4+ lymphocytes ↑	nd	pSTAT3
RF8	B	13	poor severe growth; hypereosinophilia; ESR always high; hypergammaglobulinemia (IgE)	B lymphocytes CD19+ ↓ total T lymphocytes ↑ T lymphocytes CD8+ ↑ RTE CD4+ lymphocytes ↑	SNP array	
RF9	B	8	bronchospasm at 2 years; hypereosinophilia; chronic cough; hypergammaglobulinemia (IgA)	B lymphocytes CD19+ ↑ total T lymphocytes ↑ RTE CD4+ lymphocytes ↑ CD4/CD8 ↑	nd	CD86 expression

RF12	A	3	dermatitis; enteropathy; hypergammaglobulinemia (IgA)	nd	Sequencing of *FOXP3* gene	nd
RF13	B	13	atopic dermatitis up to 2-3 years of age; punctate lesions with subsequent pus formation, accompanied by itching and scratching lesions; hypergammaglobulinemia (IgE)	RBE (CD10+ CD38+) ↑ total T lymphocytes ↑ RTE CD4+ lymphocytes ↑ B lymphocytes CD19+ switched memory ↓ CD4/CD8 ↑	nd	CD86 expression
RF14	A	18	eczema; celiac disease; autoimmune neutropenia; autoimmune thrombocytopenia	T regulatory (CD25+) ↓ DNT ab ↑	Sequencing of *FASLG* gene; WES analysis (Biodiversa)	CD86 expression
RF15	A	18	multiple lymphadenopathies; autoimmune haemolytic anemia; autoimmune neutropenia; autoimmune thrombocytopenia	T regulatory (CD25+) ↓ DNT ab ↑	clinical exome (INVITAE)	CD86 expression

RF16	A	17	arthritis; autoimmune haemolytic anemia; autoimmune thrombocytopenia; lymphoproliferation defect; splenomegaly (spleen removed + paternal familiarity for splenic lymphoma)	B lymphocytes CD19+ ↓ B lymphocytes CD19+ switched memory ↑ total T lymphocytes ↑ T lymphocytes CD8+ ↑ RTE CD4+ lymphocytes ↓	WES analysis (Biodiversa)	pSTAT3 ↑ FoxP3
RF17	A	1	hepatomegaly (linked to HLA) with increased liver enzymes; autoimmune cytopenia; lymphopenia; neonatal hypotonia; persistent increase in inflammation indices; Herpes virus infections; autoimmune thrombocytopenia; mild autoimmune neutropenia	B lymphocytes CD19+ ↑ B lymphocytes CD19+ switched memory ↓ RBE (CD10+ CD38+) ↓ total T lymphocytes ↓ T lymphocytes CD8+ ↓ T lymphocytes CD4+ ↓ RTE CD4+ lymphocytes ↓	WES analysis (Biodiversa)	pSTAT3, FoxP3 ↓, TNF, CD86 expression
RF18	A	1	autoimmune enteropathy (onset at 2 months of life with vomiting, diarrhea, fever and shock); trilinear autoimmune cytopenia (anemia, thrombocytopenia,	CD4/CD8 ↑	WES analysis (Biodiversa)	Foxp3, CTLA4

			neutropenia) hypogammaglobulinemia (IgG, IgM)			
RF19	A	3	early onset IBD; autoimmune cytopenia; lymphopenia; splenomegaly; unexplained fever	B lymphocytes CD19+ ↓ total T lymphocytes ↓ T lymphocytes CD8+ ↓ RTE CD4+ lymphocytes ↓	WES analysis (Biodiversa)	nd
RF20	A	10	autoimmune cytopenia; arthritis; mild splenomegaly; unexplained fever; recurrent infections	total T lymphocytes ↑ T lymphocytes CD8+ ↑	WES analysis (Biodiversa)	FoxP3 CD86 expression
RF21	A	3	vitiligo; autoimmune enteropathy; autoimmune thyroiditis (familiarity on the part of grandmother together with splenomegaly)	DNT ab ↑	clinical exome (INVITAE)	pSTAT3 CD86 expression
RF24	B	17	atrophic gastritis; vitiligo	T regulatory (CD25+) ↑	Panel of target genes associated with CVID	nd
RF26	B	27		-	Panel of target genes associated with CVID	nd

RF27	B	11	vitiligo; scleroderma; mild hypogammaglobulinemia (IgG)	RTE CD4+ lymphocytes ↑	Panel of target genes associated with CVID	nd
RF29	B	29	pericarditis; hypereosinophilia; atopic dermatitis; alopecia; celiac disease	RTE CD4+ lymphocytes ↑ RBE (CD10+ CD38+) ↓	Panel of target genes associated with CVID	CD86 expression

Table 8: summary of clinical and immunological classification of the 23 UDDs cases and genetic and functional analysis made to understand the cause of patients' diseases.

Evaluating the phenotype of these cases we tried to demonstrate different levels of expression for example of FOXP3, TNF-alpha or CTLA4 or different levels of phosphorylation of protein like STAT3 compared with healthy control according to what we know also from the literature. Despite some differences in immunological and functional assay we did not identify any genetic defect.

Moreover, trying to understand if our UDDs cases could be grouped each other and if abatacept could represent a valid therapeutic option also for other of our subjects, we investigated CD86 expression in B lymphocytes cells versus healthy controls in flow cytometry, thanks to what Tanaka et al explained in mice with deletion in *Tet2* and *Tet3*, where CD86 is upregulated in B lymphocyte (79). CD86 is the more present isoform in B lymphocytes of CD28 receptor for which abatacept, like CTLA4, competes to inhibit the immune response triggered by the link between CD28 and the receptor CD86.

Unfortunately, we did not see this increase expression of CD86 in our patients compared to healthy controls, rather we saw an inhibition of CD86 expression probably due to the difference between mice and human or due to the different clinical manifestations of our patients and to the pharmacological treatments that can change the pattern of protein expression of patients who are taking them (fig.33).

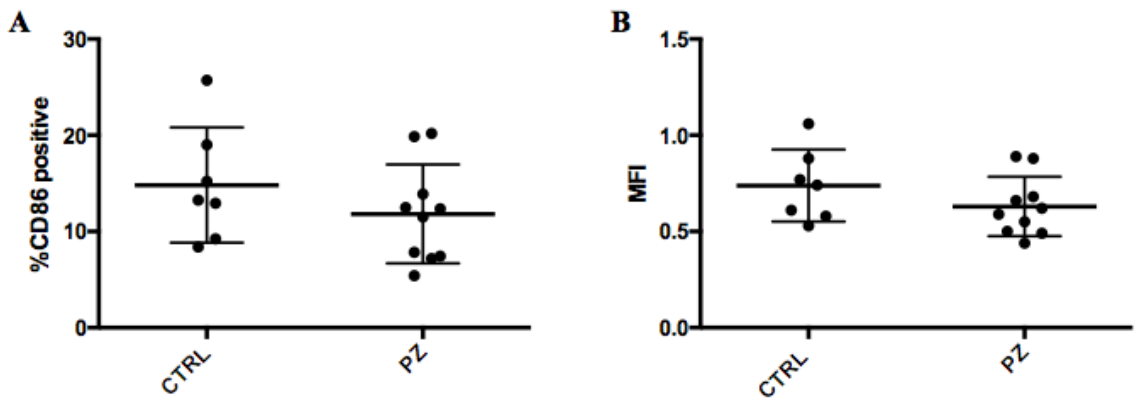


Fig.33: pattern of CD86 expression of patients (PZ) versus healthy controls (CRTL) both in terms of percentage of $CD19^{+}$ B lymphocytes positive population to CD86 (A) and of MFI (median of fluorescence) of CD86 (B).

4.4 Transcriptome analysis to subgroup UDDs cases

According to the differential gene expression pipeline, we analyzed together all the available transcriptome data of UDDs, with both positive and negative genetics (table 9) versus a group of healthy controls.

Patient	Mutation	Disease-causing gene	Related disease	Clinical Feautures
RF10	c.C6415T_p.R2139X c.C7315T_p.R2439X in *LRBA* gene	*LRBA* gene	LRBA deficiency	autoimmune gastritis; splenomegaly; multiple lymphadenopathy; hypogammaglobulinemia; anemia
RF25	c.G160A p.A54T in *CTLA4* gene	*CTLA4* gene	CTLA4 haploinsufficiency	recurrent fevers; autoimmune thrombocytopenia; autoimmune hemolytic anemia; leukopenia; recurrent infections; severe hypogammaglobulinemia
RF11	c.735ofG_p.A247Qfs*46 in *FASLG* gene	*FAS* and *FASLG* gene	ALPS	primary hypothyroidism; autoimmune enteropathy; rheumatoid arthritis; trilinear autoimmune cytopenia (haemolytic anemia, thrombocytopenia, neutropenia); recurrent infections
RF22	c.A1721C_p.N574T in *STAT1* gene	*STAT1* gene	STAT1 GOF	recurrent oral candidiasis; aphthous stomatitis (RAS); autoimmune thyroiditis, autoimmune gastritis; hemolytic anemia
RF30	c.748_750ofAAG_p.K250del in *FOXP3* gene	*FOXP3* gene	IPEX	diarrhea; atopic eczema; leukocytosis; hypereosinophilia; coeliac disease

RF17	nd	nd	nd	hepatomegaly; autoimmune cytopenia; lymphopenia; persistent increase in inflammation indices; Herpes virus infections; autoimmune thrombocytopenia;
RF19	nd	nd	nd	early onset IBD; autoimmune cytopenia; lymphopenia; splenomegaly; unexplained fever

Table 9: UDDs cases with available transcriptome data.

In the PCA analysis (fig.34) which explain the gene expression variability across all samples, it is possible to notice that patient with LRBA deficiency clusterized close to patient with IPEX and FASLG mutation, an aspect that confirms the definition of LRBA deficiency as ALPS-like disease or IPEX-like disease (20, 58, 62, 66, 80-82). Moreover, this similarity was highlighted even more by the clusterization near two ALPS cases of two brothers both with mutations in the *FAS* gene. Moreover, the patient withSTAT1 GOF localized near the LRBA defect. It is not well understood the gene expression similarity between these two patients, however it was atypical also the results from flow cytometry analysis in which STAT1 expression was not clearly increased compared to control. Moreover, we could observe the stratification of two of our genetic negative UDDs cases in comparison to the diagnosed monogenic defects and healthy controls and it was very interesting especially for the patient that clusterizes close to CTLA4 haploinsufficiency. This is the case of a 3 years old child with a clinical picture of early onset IBD, autoimmune cytopenia, lymphopenia of both T and B cells population, splenomegaly and unexplained fever. The WES analysis did not reveal any variants that could explain this phenotype, but due to the results of transcriptome analysis we are planning to analyze by flow cytometry the surface expression of CTLA4 and especially the phosphorylation levels of S6 according to the previously obtained results performed on cells of patients with mutation in CTLA4 and in LRBA.

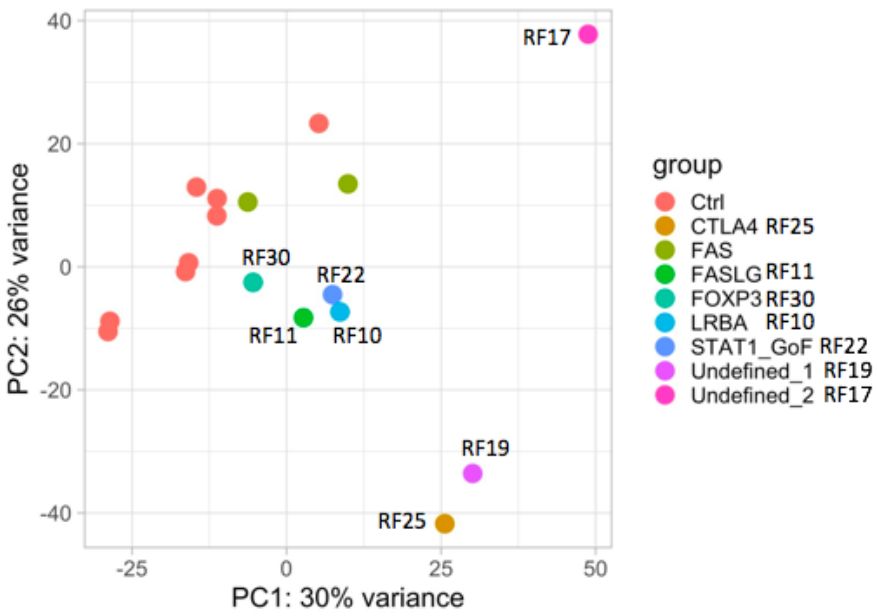


Fig.34: distribution of patients and healthy controls according to the 5000 most variable 5 genes.

5. CONCLUSION

In these 3 years we enrolled and clinical characterized 30 patients, 15 meeting criteria A, 12 meeting criteria B and 3 overlapping both criteria. We studied immune dysregulation diseases in our patients exploiting data from clinics, immunophenotype (lymphocyte subpopulations analyzed by flow cytometry) and genetic investigations. Subsequently, we checked if a comparison between cases with and without a definite genetic cause could reveal similarities between the two groups, possibly relevant to the involved pathogenic mechanisms (table 10).

Patient	Genetic analysis	Immuno phenotype	Functional assay	Transcriptomic analysis	Mutations discovered
RF1	-	✓	-	-	-
RF2	-	✓	-	-	-
RF3	WES	✓	STAT3 and STAT5 phosphorilation	-	-
RF4	-	✓	-	-	-
RF5	WES	✓	CD86	-	-
RF6	-	✓	-	-	-
RF7	-	✓	STAT3 and STAT5 phosphorilation	-	-
RF8	-	✓	-	-	-
RF9	-	✓	CD86	-	-
RF10	Panel of genes	✓	CD86, CTLA4 expression, LRBA expression, S6 phosphorilation	✓	c.C6415T_p.R2139X c.C7315T_p.R2439X in LRBA gene
RF11	WES	✓	S6 phosphorilation	✓	c.735ofG_p.A247Qfs*46 in *FASLG* gene
RF12	WES	-	-	-	-
RF13	-	✓	CD86	-	-
RF14	WES	✓	CD86	-	-
RF15	Invitae panel	✓	CD86	-	-
RF16	WES	✓	STAT3 and STAT5 phosphorilation, FOXP3 expression	-	-
RF17	WES	✓	CD86, STAT3 and STAT5 phosphorilation, FOXP3 expression, TNFα expression	✓	-
RF18	WES	✓	CTLA4 expression, FOXP3 expression	-	-
RF19	WES	✓		✓	-
RF20	WES	✓	CD86, FOXP3 expression	-	-
RF21	Invitae panel	✓	CD86, STAT3 phosphorilation	-	-
RF22	Panel of genes	✓	STAT1 expression	✓	c.A1721C_p.N574T in *STAT1* gene
RF23	Invitae panel	✓	-	-	-
RF24	Panel of genes	✓	-	-	-
RF25	Invitae panel	✓	CD86, CTLA4 expression, S6 phosphorilation	✓	c.G160A p.A54T in CTLA4 gene
RF26	Panel of genes	✓	-	-	-
RF27	Panel of genes	✓	-	-	-
RF28	Panel of genes	✓	CTLA4 expression, S6 phosphorilation	-	-
RF29	Panel of genes	✓	CD86	-	-
RF30	WES	✓	-	✓	c.748_750ofAAG_p.K250del in *FOXP3* gene

Table 10: summary table of performed analysis and obtained results of all 30 enrolled patients

According to this combined analysis strategy, 7 patients received a genetic diagnosis, whilst 23 remained Undefined Dysregulatory Disorders (UDD).

- LRBA deficiency and CTLA4 haploinsufficiency diagnosed in 3 patients with autoimmune phenotype (recruitment criteria A);
- IPEX syndrome due to mutation in FOXP3 gene was found in a patient with predominantly allergic phenotype (recruitment criteria B);
- mutations in FASLG, STAT1, TNFRSF13B gene, associated with ALPS disease, STAT1 GOF and CVID respectively were observed in 3 patients with allergy criteria together with autoimmune manifestations.

Where possible, functional assays were performed to estimate the significance of the discovered variants and to explain the complex clinical picture in the 7 patients with monogenic findings. In these cases, we also performed the investigation of available transcriptome profiles.

Furthermore, the clinical, immunological and genetic profiles were classified and combined, and functional assays were also carried out to clarify their sets of symptoms and to improve therapeutic treatments, but without any successful results especially from a genetic point of view.

By analyzing the entire series, we can notice that some symptoms, manifested in patients who received a monogenic diagnosis, occurred also in patients with negative genetic results, raising the question whether they could share the involvement of the same pathogenic pathways. Of note, we could observe how the transcriptomes of two of these still undefined patients clustered when compared with those of patients with definite monogenic disorders and healthy controls. RF19 clustered near CTLA4 haploinsufficiency, inducing us to perform functional assays of CTLA4 expression analysis and S6 phosphorylation levels analysis in flow cytometry, which however tested normal. These results may suggest that the observed similarities between the two transcription profiles can be due to the sharing of other pathogenic features.

As a future perspective, we will compare the transcriptome data of our patients with other cases of PID collected outside of this study. Moreover, by expanding our data we could be able to make a multivariate analysis for each of the 3 categories.

One characteristic of several patients included in our study concerned their referral to multiple specialties, because of complex clinical pictures encompassing various organ involvement. We

highlight that this behavior may be suspicious of a dysregulatory PID and should warrant proper immunological and genetic investigations, in particular in pediatric age (fig.35). This is even more important as a prompt diagnosis of some of these dysregulatory PIDs can pave the avenue for precision treatments, which may prevent a worse disease progression. Moreover, it is also worth increasing awareness of the diagnostic and therapeutic opportunities in these patients, who should be cared for with the collaboration of referral centers where functional assays and genetic capabilities are integrated with immunological skills.

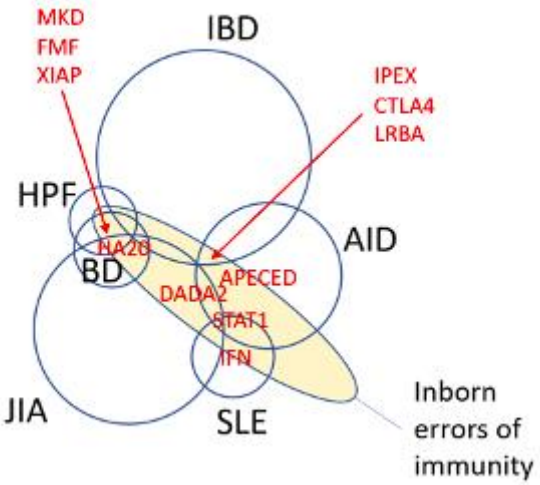


Fig.35: Some druggable IEIs in areas of intersection between more common gnoseological entities: in red druggable IEIs and in black common gnoseological entities. JIA: Juvenile Idiopathic Arthritis; BD: Bowel Disease; A20: A20 haploinsufficiency; HPF: hereditary periodic fever; IBD: Inflammatory Bowel Disease; CTLA4: Cytotoxic T-Lymphocyte Antigen 4; IPEX: Immunodysregulation polyendocrinopathy enteropathy X-linked; LRBA: lipopolysaccharide (LPS)-responsive and beige-like anchor protein; STAT1: Signal transducer and activator of transcription 1; SLE: Systemic Lupus Erythematosus

6. SUPPLEMENTARY MATERIALS

6.1 Ficoll

To evaluate intracellular expression of our proteins of interest we separated PBMCs of patient and healthy control from peripheral whole blood in heparin, stratifying through centrifugation based on density gradient Ficoll (1,077 g/mL, Lympholyte, Cederlane) (fig.36).

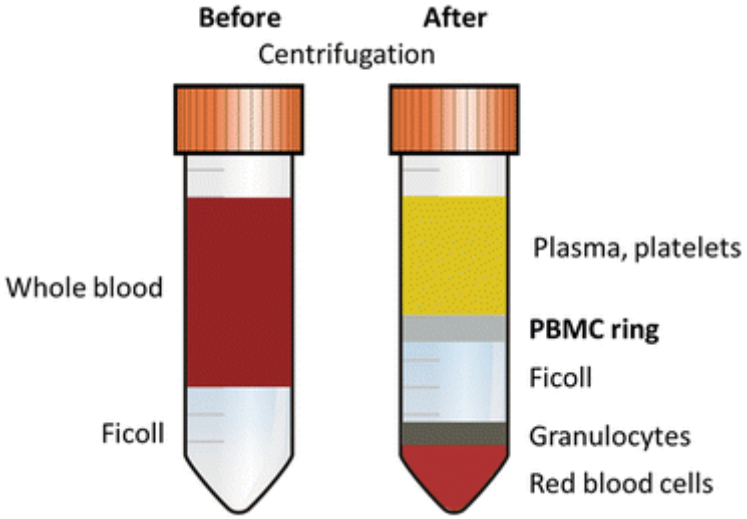


Fig.36: PBMCs separation from peripheral whole blood through Ficoll technique.

6.2 CTLA4 expression

2×10^5 PBMCs of patient and healthy control at the density of 1×10^6 cells/mL in RPMI, 10% HS-AB, P/S-L-Glu for each condition, untreated and stimulated with PMA and ionomycin (Cell Stimulation Cocktail 500X, Invitrogen) for 2 hours at 37°C, 5% CO_2 or with PHA (5ug/mL, Biochrom AG) for 24 hours at 37°C, 5% CO_2.

Cells were stained with CD3 VioBlue (Miltenyi), CD4 APC (BD) and CTLA4 PE (BD) to analyze CTLA4 expression in $CD4^+$ T cells and in resting lymphocytes by flow cytometry.

Sample data were acquired using MACSQuant Analyzer 10 (Miltenyi Biotec) and were analysed with FlowLogic software (version 7.2.1, Inivai Technologies).

6.3 LRBA expression

We resuspended at least 1×10^6 PBMCs at the density of 1×10^6 cells/mL in RPMI, 10% FBS, P/S-L-Glu for each condition, untreated and stimulated with PHA (1ug/mL, Biochrom AG) and incubated 72 hours at 37°C, 5% CO_2.

Moreover, 250.000 cells from patient and ctrl were first collected and wash in saline at 300xg, 10', RT and stained with surface antibodies CD3 REA613 (VioBlue, Miltenyi), CD4 APC (BD) and CD69 (PE-Vio770, Miltenyi). Then cells have been fixed and permeabilized with Fix&Perm ADG kit and marked before with anti-LRBA antibody (Sigma) for 30 minutes and then with secondary antibody (BD) for another 30 minutes. Samples were analyzed by flow cytometry MACSQuant Analyzer 10 (Miltenyi Biotec) and were analysed with FlowLogic software (version 7.2.1, Inivai Technologies) to observe the starting point of cells for LRBA expression without stimulation at time zero.

The same protocol was made at the end of 72 hours of stimulation, both for untreated and cells stimulated with PHA.

CTLA4 expression was analyzed in $CD3^+CD69^+$ T cells and in resting lymphocytes by flow cytometry.
Sample data were acquired using MACSQuant Analyzer 10 (Miltenyi Biotec) and were analysed with FlowLogic software (version 7.2.1, Inivai Technologies).

6.4 S6 phosphorylation

4×10^5 PBMCs of patient and healthy control have been resuspended in at the density of 1×10^6 cells/mL in RPMI, 10% HS-AB, P/S-L-Glu for each condition, untreated and stimulated with Dynabeads Human T-activator CD3/CD28 (Gibco by Life Technologies) for 4 hours at 37°C, 5% CO_2.
Cells were surface stained with CD4 APC (Miltenyi) and CD3 VioBlue (Miltenyi) and then fixed and permeabilized with paraformaldehyde 4% () and methanol 90% respectively to stain with anti-pS6 PE (Cell Signaling) or corresponding isotype PE (Cell Signaling). The level of S6 phosphorylation was analyzed in CD4+ T cells activated in flow cytometry.
Sample data were acquired using MACSQuant Analyzer 10 (Miltenyi Biotec) and were analysed with FlowLogic software (version 7.2.1, Inivai Technologies).

6.5 STAT1 expression

5×10^5 PBMCs of patient and healthy control have been resuspended in at the density of 1×10^6 cells/mL in RPMI, 5% HS-AB, P/S-L-Glu for each condition, untreated and stimulated with IFNα2a (300 U/mL, Miltenyi Biotech) for 24 hours at 37°C, 5% CO_2.

Cells were surface stained with CD3 VioBlue (Miltenyi), CD4 PE (Miltenyi) and CD45 V500-C (BD). Then cells have been fixed and permeabilized with Fix&Perm Biolegend kit to stain with anti-STAT1 AF647 (BD) or corresponding isotype ctrl AF647 (Biolegend).
The expression level of STAT1 was analyzed in CD4+ T cells in flow cytometry.
Sample data were acquired using MACSQuant Analyzer 10 (Miltenyi Biotec) and were analysed with FlowLogic software (version 7.2.1, Inivai Technologies).

6.6 STAT3 phosphorylation

5×10^5 PBMCs of patient and healthy control have been resuspended in at the density of 1×10^6 cells/mL in RPMI, 5% HS-AB, P/S-L-Glu for each condition, untreated and stimulated with IL-6 (100 ng/mL, Miltenyi) for 20 minutes at 37°C, 5% CO_2.
Cells were surface stained with CD3 VioBlue (Miltenyi), CD14 FITC (Dako) and then fixed and permeabilized with paraformaldehyde 4% () and methanol 90% respectively to stain with anti-pSTAT3 AlexaFluor 647 (BD) or corresponding isotype AlexaFluor 647 (Biolegend). The level of STAT3 phosphorylation was analyzed in CD14+ cells in flow cytometry.
Sample data were acquired using MACSQuant Analyzer 10 (Miltenyi Biotec) and were analysed with FlowLogic software (version 7.2.1, Inivai Technologies).

6.7 FOXP3 expression

5×10^5 PBMCs of patient and healthy control have been resuspended in 100uL of saline solution and surface stained with CD3 VioBlue (Miltenyi), CD45 V500-C (BD), CD4 APC (BD), CD25 PE (BD). Then cells have been fixed and permeabilized with Human FoxP3 Buffer Set (BD Pharmingen) to stain with anti-FoxP3 AF488 (BD) or corresponding isotype control IgG1 AF488 (BD).
The expression level of FoxP3 was analyzed in CD4+ cells in flow cytometry.
Sample data were acquired using MACSQuant Analyzer 10 (Miltenyi Biotec) and were analysed with FlowLogic software (version 7.2.1, Inivai Technologies).

6.8 Real-time PCR

Real-time PCR was performed on whole blood collected in PaxGene blood RNA tubes (PreAnalytiX, Quiagen/BD company) and the RNA was extracted with corresponding PaxGene blood RNA kit (PreAnalytiX, Quiagen/BD company). The gene evaluated was *LRBA* and *FASLG* and their expression were assessed using AB 7500 Real Time PCR System

(Applied Biosystems,Waltham, MA, USA), TaqMan Gene Expression Master Mix (Applied Biosystems) and Taqman gene expression probes (Thermo Fisher). Using AB 7500 Real Time PCR software, each target quantity was normalized with the expression level of two housekeeping gene, *HPRT1* and *G6PD*, and the relative quantification was conducted relating to the calibrator sample (mix of RNA controls) using the $2^{-\Delta\Delta Ct}$ method (83).

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