Autoimmune Lymphoproliferative Syndrome (Alps) Disease and Alps Phenotype: Are They Two Distinct Entities?

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Abstract

Introduction

Autoimmune Lymphoproliferative Syndrome (ALPS) is an inherited disorder of lymphocyte homeostasis classically due to mutation of FAS, FASL, and CASP10 genes (ALPSFAS/CASP10). In spite of recent progress, about one third of ALPS patients does not carry classical mutations and still remains gene orphan (ALPS-U, undetermined genetic defects).

Objective

The aims of the present study were to compare the clinical and immunological features of ALPSFAS/CASP10 vs those of ALPS-U affected subjects and to deepen the genetic characteristics of this latter group.

Methods

Demographical, anamnestic, biochemical data were retrieved from medical record of 42 ALPS subjects. An enlarged panel of genes (Next Generation Sequencing) was applied to the ALPS-U group.

Results

ALPS-U subjects showed a more complex phenotype if compared to ALPSFAS/CASP10 group, characterized by multi-organ involvement ($p=0.001$) and positivity of autoimmune markers ($p=0.02$). Multilineage cytopenia was present in both groups without differences with the exception of lymphocytopenia and autoimmune neutropenia that were more frequent in ALPS-U than in ALPS-FAS/CASP10 group ($p=0.01$ and $p=0.04$). First and second line treatments were able to control the symptoms in 100% of the ALPSFAS/CASP10 patients, while 63% of ALPS-U needed more than two lines of treatment and remission in some cases was obtained only after target therapy. In the ALPS-U group, we found in 14/28 (50%) patients 19 variants; of these 4/19 (21%) were known as pathogenic, 8/19 (42%) as likely pathogenic. A characteristic flow cytometry panel including CD3CD4-CD8+TCR $\alpha\beta$ +, CD3+CD25+/CD3HLADR+, TCR $\alpha\beta$ + B220+ and CD19+CD27+ identified the ALPS FAS/CASP10 group.

Introduction

Autoimmune Lymphoproliferative Syndrome (ALPS) is a rare inherited disorder due to the dysregulation of the FAS apoptotic pathway, usually manifested in childhood, though symptoms may occur at any age. As a result of disturbed lymphocyte homeostasis, auto-reactive cells persist,

leading to development of chronic non malignant lymphadenopathy, splenomegaly, multi-lineage cytopenias, and increased risk of lymphoma [1]. Currently the diagnosis of ALPS is based on phenotypical criteria since is determined by the combination of different clinical and laboratory findings as from reported in 2009 by a panel of NIH experts [2]. Lymphoproliferation is the most common clinical manifestation of ALPS followed by autoimmunity that appears in 70% of cases [3, 4]. Many patients have isolated or variable combination of immune cytopenia such as Autoimmune Hemolytic Anemia (AIHA), Neutropenia (AIN) and Immune Thrombocytopenia (ITP) [5].

The typical biomarker is an increased number of a characteristic T-cell population named "double negative T cells" (DNTs); other characteristic laboratory abnormalities include elevated levels of interleukin (IL) 10, IL-18, vitamin B12, soluble FAS ligand (sFASL) [6].

Elevated DNTs lymphocytes expressing B220 [7, 8, 9], together with an expansion of HLA-DR+ T cells [10] and a decrease of CD4+CD25+ T-cells and CD27+ B cells [10, 11] were also reported in ALPS patients and generated the proposal of a flow-cytometry panel as a diagnostic screening tool for ALPS including: CD3CD4-CD8+TCR $\alpha\beta$ +, CD3+CD25+/CD3HLADR+, TCR $\alpha\beta$ + B220+ and CD19+CD27+.

Management of ALPS patients aims to control clinical manifestations and specific complications like cytopenia are often difficult to handle since stable remission may be difficult to achieve after the first (steroids and immunoglobulins) and even the second line of treatment (Mycophenolate-mophetil, MMF and rapamycin) [12, 13, 14, 15, 16].

Mutations in FAS, FASL, and CASP 10 genes are found to cause the disease in about 70% of patients, but in one third of ALPS subjects no pathogenic genetic lesion is found (ALPS-U, undetermined genetic defects) [2, 14, 17].

Discovery of novel defects causing immunodeficiencies or immune-dysregulation would be identified as potential drivers in ALPS-U patients [18]. ALPS, which is primarily a "phenotypic definition" according to the NIH criteria, as a matter of fact, probably hide different diseases raising the point of additional specific mechanisms that can be targeted by effective therapies [19].

The aim of the present study is to compare the two groups ALPS-FAS/CASP10 vs ALPS-U in order to identify any possible surrogate markers that may direct the diagnosis towards more precise genetic categories.

Patients And Methods

Patients

Patients were defined as ALPS definitive and probable according to the criteria established by the NIH in 2009 [2]. Demographic data, clinical findings, biochemical markers (vitamin B12, interleukin 10 and interleukin 18, sFASL, immunoglobulins serum level, lymphocyte subsets analysis including CD3+CD4-CD8- TCR $\alpha\beta$ +, named double negative T Cells-DNT cells), autoimmune markers (anti-platelet and anti neutrophil indirect antibodies, direct and indirect Coombs test, ANA, ENA, ASMA, anti dsDNA, anti gliadin, anti transglutaminase, anti endomysium and antitireoglobulin antibodies) were entered into an electronic national ALPS database (ALPS Italian Network).

All the information were retrieved from the medical records of patients after informed consent according to the Helsinki declaration of principles. Patients were referred at the ALPS Italian network through the AIEOP (Italian Pediatric Hemato-Oncology Association) centers.

Response to therapy was evaluated after at least 4-6 weeks of drug administration for each of the following clinical symptoms: cytopenia, lymphoproliferation, fever or other autoimmune symptoms and scored as complete (CR), partial (PR) or non response (NR). (See for further detail the footnotes of table 3). In keeping with the response criteria from our previous publication [19] a patient was considered as Complete Responder if all the aforementioned clinical symptoms disappeared/normalized 4-6 weeks after treatment start. A patient was considered as Partial Responder if at least one of the aforementioned clinical symptom improved/normalized 4-6 weeks after treatment start. A patient was considered as Non Responder if none of the above symptoms improved/normalized 4-6 weeks after treatment start.

First line treatment was steroids or intravenous immunoglobulins whereas second line was mycophenolate-mophetil (MMF) or rapamycin. Further lines of treatment included: Rituximab plus MMF or rapamycin, Anakinra, cyclosporine A, Eltrombopag or Nplate (whenever cytopenia was the dominating sign) either alone or in combination with MMF or rapamycin.

Lymphocyte immunophenotypes

A flow-cytometry panel including four parameters suggestive of ALPS (CD3CD4-CD8+TCR $\alpha\beta$ +>1,5% of total lymphocytes, CD3CD25+/CD3HLADR+<1%, TCR $\alpha\beta$ + B220+>60 % of DNT and CD19+CD27+< 15% of B cells) [7,11] was used as immunological screening. Peripheral lymphocyte subsets were evaluated from whole blood using an eight-color immunostaining panel (lyse and wash procedure), a FACS Canto II flow cytometer (BD) equipped with three lasers (blue, red, violet), FACSDiva™ software (BD), and a large panel of RUO mAbs and fluorochromes variously combined (all BD).

FAS mediated apoptosis test

Fas-induced cell death was evaluated on T cell lines obtained by activating PBMCs with PHA at days 0 (1 μ g/ml) and 12-15 (0,1 μ g/ml) and cultured in RPMI 1640 + 10% FCS + rIL-2 (2 U/ml). Fas function was assessed 6 days after the second stimulation (day 21). Cells were incubated with control medium or anti-Fas mAb (CH11, IgM isotype) (1 μ g/ml) in the presence of rIL-2 (1 U/ml) to minimize spontaneous cell death. Cell survival was evaluated after 18 h by counting live cells in each well by the trypan blue exclusion test and by flow cytometry of cells excluding propidium iodide. Cells from two normal donors were included in experiment as positive controls. Results were expressed as specific cell survival % calculated as follows: (total live cell count in the assay well/total live cell count in the control well) x 100. Fas function was defined as defective when cell survival was >78% (the 95th percentile of data obtained from 200 normal controls). [20]

Interleukin 18, Interleukin 10, and FasL

Serum interleukin 18, interleukin 10, and FasL assays were performed using commercially available ELISA (Enzyme-Linked Immuno Sorbent Assay) kits (MBL, Woburn, MA, USA, Invitrogen, Waltham, MA, USA, Abnova, Taipei, Taiwan, respectively).

Genetic analysis

Forthy six ALPS-FAS/CASP10 and ALPS-U patients underwent genetic analysis. Molecular variants were detected by Sanger sequencing in the less recent diagnoses and specifically 10/18 subjects of the ALPS FAS/CASP10 group and 3/28 of the ALPS-U group or by Next Generation Sequencing techniques (NGS) with Sanger confirmation (the most recent diagnoses; 8/18 patients of the ALPS FAS/CASP10 and 25/28 of the ALPS-U group).

Analysis were carried out on peripheral blood and not on sorted DNT cells.

The HaloPlex Target Enrichment System, was used for the NGS panel (Version C1, December 2016; Version E1, July 2015) and included 315 genes involved in haematological disorders, immunodeficiencies, immune-dysregulation, inflammatory and bone marrow failure syndromes.

Raw data were analyzed and variants assessed by the Ion Reporter™ Software 5.0 (<https://ionreporter.thermofisher.com/ir/>). To confirm the presence of the variants thus selected Sanger PCR protocol was set up for each variant to confirm genetic mutations detected by NGS. Functional studies were performed to assess defects in the proteins encoded by the detected variants. Apoptosis induced by FAS-ligand and TRAIL stimulation was assessed on patient EBV-immortalized B-cells. Apoptosis pathway function was evaluated by western-blot analysis of CASP10, CASP8, and PARP proteins using rabbit monoclonal anti-Caspase-10 antibodies (Abcam), mouse monoclonal anti-Caspase-8 (1C12), and rabbit polyclonal anti-PARP (Cell Signaling). In one case quantitation of the protein (ADA2) encoded by the defective gene was performed on fresh blood.

Statistical analysis

Descriptive statistics were reported in terms of absolute frequencies and percentages. Distribution of data regarding continuous variables were described in terms of a median value and range. Comparison of frequency distribution was analyzed by Chi-square test. Fisher's exact test was

used in case of at least one expected frequency less than five. Comparison regarding demographic characteristics and double negative values was analyzed by Mann Whitney test as non parametric test. All test were two-tailed and *P* value less than 0.05 was considered statistically significant.

STATA statistical software (release 7.0, StataCorp 2001, College Station, TX, USA) was used.

Results

Charateristic of the whole cohort

Forthysix patients (20 females, 43%) diagnosed with ALPS referred at the ALPS Italian network through the AIEOP centers from 2002 to 2019, were considered eligible for the present study.

Thirtyseven of 46 (81%) were affected with definitive ALPS and 9/46 (20%) with probable ALPS according the 2009 NIH criteria [2].

The median age at clinical onset was 6 years (range 0-23 years) and median age at diagnosis was 12 years (range 1 to 39 years). Median duration of follow-up of the whole cohort was 3,3 years (range 0-14 years). Patients were genetically tested in order to differentiate classical ALPS

(FAS/CASP 10 mutated) from ALPS-U (those negative for FAS/CASP10 variants). Then ALPS FAS/CASP10 and ALPS-U subjects were compared by several clinical, biochemical and flow citometry markers and by treatment response.

Genetic testing

Genetic testing applied to the whole cohort of 46 subjects showed that 18 patients (39%) carried the classical FAS/CASP10 gene mutations 12 with FAS and 6 with CASP10) while 28 patients (61%) were negative for these mutations and were identified as ALPS-U (undetermined genetic defects).

In the ALPS FAS/CASP10 group 11 variants were known as pathogenic/likely pathogenic (Table 1). The two CASP10 p.Tyr446Cys and CASP10 p.Val410Ile variants found in Pt 15, 16 and 18 respectively, known as benign were found to be associated to impaired apoptosis driven by FAS-ligand and TRAIL stimulation [21] (Table 1). The remaining 4 variants are considered VUS or likely benign or benign.

Within the ALPS-U group, we found in 14/28 (50%) patients 19 variants; of these 4/19 (21%) were known as pathogenic, 8/19 (42%) as likely pathogenic and 4/19 (21%) as VUS. In one patient (ALPS-U Pt 23) we found two CECR1 gene variants, reported as VUS/likely pathogenic, that was associated to reduced expression (assessed in Western blot) of the corresponding ADA2 protein (Table 1).

Clinical, biochemical and flow cytometry markers

The phenotypes of the 46 ALPS-FAS/CASP10 and ALPS-U patients were compared by a large number of clinical and biological parameters (table 3).

As expected, chronic (<6mo) non malignant lymphoproliferation expressed by lymphadenopathy and or/splenomegaly [14,22], was present in all patients in both groups. Lymphoadenopathy and splenomegaly was contemporarily present in 67% ALPS-FAS/CASP10 group and in 54% ALPS-U group (*p*=0.37).

Cytopenia affecting one or more haematopoietic lineages without difference in distribution between ALPS-FAS/CASP10 and ALPS-U 81% vs 82%; *p*=*ns*) groups.

Thrombocytopenia was the most frequent cytopenia in both groups (85% vs 83%; *p*=*ns*) whereas lymphocytopenia and autoimmune neutropenia were most commonly seen in ALPS-U (12/28 and 15/23; 43% and 65%) than in ALPS-FAS/CASP10 group (1/16 and 4/13; 6% and 31%) (*p*=0.01 and *p*=0.04).

A flow-cytometry panel identifying four specific lymphocyte subpopulations (CD3CD4-CD8+TCRαβ+, CD3+CD25+/CD3HLADR+, TCR αβ+ B220+and CD19+CD27+) and proposed as a diagnostic tool for screening ALPS [23], was compared in ALPSFAS/CASP10 vs ALPS-U. When taken individually, each of these lymphocyte subpopulation was not significantly different in the two groups. However, when taken together, the whole pathologic panel was significantly associated (*p*=0.01) with ALPS-FAS/CAS10 thus suggesting that the contemporary positivity of these parameters might be a hallmark for ALPS-FAS/CASP10 and useful for differentiating from ALPS-U (Table 4).

Increased vitamin B12 serum levels (>1500 mg/dL) were significantly more frequent in ALPS-FAS/CASP10 than in ALPS-U (*p*=0.001) and so was IL10 (>20pg/mL) (*p*=0.002) (Table 3).

Not surprisingly, Double Negative T cells (DNT), the primary hallmark of ALPS, were not significant different in ALPS-FAS/CAS10 population (median value of 3.9% (range 1.9%-13%) as compared to ALPS-U (median value of 2.6%; range 1.7%-14.8%;(*p*=0.11).

No patients were found to have somatic mutations on FAS gene. This may reflect a lack of the sequencing assay, because genetic analysis was not performed on sorted DNTs.

FAS-mediated apoptosis test has been done in all subjects of ALPS FAS/CASP10 (1 failed) and in 27/28 of ALPS-U group. The comparison was not statistically significant.

Overall, markers of autoimmunity were positive in 6/18 (33%) subjects of the ALPS-FAS/CASP10 group and in 19/28 (68%) ALPS-U subjects (*p*=0.02). In terms of frequency, Direct Antibodies Test (DAT+) was the most common positive marker in ALPS-FAS/CASP10 group (4/14; 28%); while anti-Nuclear Antibodies (ANA) were so in ALPS-U group (7/28; 25%).

Signs and symptoms of autoimmune diseases (arthritis, coeliac disease, primary sclerosing cholangitis, thyroiditis, autoimmune hepatitis, oral ulcers, chronic inflammatory GI diseases, type 1 diabetes, nephrotic syndrome and eczema) were significantly more frequent in ALPS-U (21/28, 75%) than in ALPS-FAS/CASP10 patients 4/18, 22%) ($p=0.001$).

Treatment

Three out of 46 (6%) patients did not require any treatment, while the remaining 43 (93%) received one or more lines or drug combinations. First line treatment (steroid or intravenous immunoglobulins) was effective in only 13/38 subjects (30%), 6/13 (46%) in ALPS FAS/CASP10 and 7/22 (32%) in ALPS U group ($p=0.39$).

The response rate to second-line (MMF or rapamycin) was 100% (12/12) in ALPS FAS/CASP10 and 33% (7/21) in ALPS-U group ($p=0.0001$). In the ALPS-U patients not responding to second line therapy, further lines of treatment were needed. Abatacept (a recombinant fusion protein-drug that mimics the action of CTLA4), was successfully administered in two LRBA mutated patients. In the remaining ALPS-U subjects various combination treatments were adopted (Rituximab, Anakinra, Eltrombopag or Nplate, either alone or in combination with MMF or rapamycin). In ALPS-U group, a complete response was achieved in 12/27 (44%) and a partial response in 10/27 (37%).

Overall ALPS-U received a significantly higher number of combination treatments (n° 17/27, 63%) over ALPS-FAS/CASP10 (n° 1/16, 6%) ($p=0.01$) and a significantly greater number of drugs (14/27 (52%) vs 2/16 (12%) of ALPS-FAS/CASP10 to achieve a response ($p=0.009$) (table 3).

Discussion

In this study we report on the largest comparison of classical ALPS (FAS/CASP10) with ALPS-U patients, diagnosed according to the NIH 2009 criteria [2]. The analysis shows that a consistent number of ALPS-U patients display a distinct clinical and biological phenotype and also have a different genetic background when compared to ALPS FAS/CASP10 subjects.

Indeed the clinical phenotype of ALPS-U patients appeared to be characterized by and increased frequency of lymphocytopenia, of associated autoimmune makers/symptoms and consequently of multiple organ involvement and by a greater difficulty in achieving response as expressed by the need for more lineages of treatments and drug combinations with a far inferior response to MMF and to rapamycin (the second line treatment) vs ALPS FAS/CASP10 subjects. Incidentally, although the numerosity of the sample size is relatively limited, it has to be noted the higher response rate achieved in classical ALPS patients with second line treatment MMF or rapamycin compared to first line therapies steroids or immunoglobulins.

This highlights the option for MMF or rapamycin to shift to up-front therapies in documented classical ALPS [24].

Also biological phenotype was different from ALPS FAS/CASP10 since ALPS-U patients displayed a significantly lower association with elevated VitaminB12 and IL 10 serum levels.

Another important difference is related to the flow-cytometry panel proposed in past years as a screening for ALPS diagnosis [23]. Indeed in our cohort, this panel was significantly associated to ALPS-FAS/CASP10 when all the four parameters were contemporarily positive. Although this may somehow orient clinicians towards ALPS FAS/CASP10 vs ALPS-U. It has to be outlined that the final diagnosis can only rely on genetic analysis.

The percentage of 64% of sharing the quadruple positivity of immunophenotype elects in ALPSFAS/CASP10 group, probably delineates a peculiar characteristic partially represents in ALPS-U patients surely including a portion of overlap.

In classical studies [2,14,17] ALPS patients were divided into two groups based on the genetic background: those in whom a pathogenic mutations in FAS, FASL, and CASP10 genes were found, and those, named ALPS-U and accounting to up to one third of the total, in whom no pathogenic genetic lesion was detectable [12,13]. In our study we found that ALPS-U subjects represented the majority (61%) of the whole cohort. Reason for this difference is not obvious and might possibly be related to selection bias inherent in Registry studies. However, thanks to NGS resources, genetic variants were found in about half (14/28) of ALPS-U patients (Table 1). The pathogenic role of the detected mutations was known only for a minority of them (4/14). In one additional patient (ALPS-U Pt 23) we found a CECR1 gene variant (p.Leu188Pro; p.Thr187Pro) reported as likely pathogenic. Indeed we proved that this variant caused the reduced expression of the corresponding protein ADA2 thus supporting the idea of a pathogenic role for this variant. In the ALPS FAS/CASP10 all subjects carrying benign/likely benign variants of CASP10, an altered apoptosis induced by FAS-ligand and TRAIL stimulation was show thus suggesting a pathogenic role for these variants too [21]. The variants of CASP10 have always been reported in literature with conflicting interpretation; nevertheless some of them have been recently reported to be relate to impair apoptosis and suggest that could play a role in predisposition of immune-dysregulation [21].

The remaining patients either carried variants considered as probably pathogenic or as VUS.

Variants of unknown significance (VUS) have a probable effect on the phenotype. Though taking VUS variants into consideration for diagnostic purpose may be questionable; however, as clinicians, found VUS in patients showing symptoms and laboratory alterations similar to ALPS FAS/CASP10 patients, may have an impact. [25]

If we consider the whole cohort it is worth noting that virtually all of detected variants are related to immune-deficiencies/immune-dysregulations diseases. Interestingly the same was shown in a recent study on large cohort of pediatric Evans syndrome [26] patients where an association was found with high frequency of potentially damaging variants in immune genes.

The availability of the new genetic techniques like NGS and WGS, will probably modify the "classical genotype/phenotype associations" widening the clinical variability referred to a single gene variant as recently described for LRBA and CTLA4 deficient patients [27,28]. Nevertheless, still this with standing the need

remains for functional validation analyses aimed to prove the real pathogenic role of likely pathogenic variants or VUS.

Overall our study suggests that the ALPS-FAS/CASP10 represents a well defined disease with specific genetic background, rather definite clinical signs and immunological markers and a rather predictable response to treatment that appears to be optimal to MMF or rapamycin. On the contrary ALPS-U, that differs from ALPS-FAS/CASP10 because it has a clearer non haematological autoimmune signature, it is more associated to lymphopenia, to lower response to treatment in general and to drugs classically effective in ALPS-FAS/CASP10 like MMF and rapamycin, appears to be a more heterogeneous group often generated by pathogenic variants of immunodeficiency or immune-dysregulation genes.

Although some genetic diagnosis that we detected correspond to well defined disorders different from ALPS (like for example CTLA4 and LRBA) we think that this study does not diminish the role of genetic analysis, but rather outlines the need to revise ALPS diagnostic criteria. On the contrary, this study envisages the importance to apply enlarged genetic panels to ALPS-U patients including several immunodeficiencies and immune-dysregulation genes and to recur to WES analysis when a pathogenic gene is not found and to always look at genetic findings in the context of the clinical phenotype.

Declarations

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Authorship Contributions

All authors contributed to the study conception and design. Elena Palmisani: designed the research, contributed essential data, cared for the patients and wrote the manuscript. Alice Grossi, Isabella Ceccherini, Marina Lanciotti: performed the genetic analysis. Michela Lupia: performed the IL-18, IL-10 and FAS ligand tests. Palola Terranova: performed the study of lymphocyte immune phenotype. Francesca Tosetti: performed functional tests. Nicoletta Crescenzo: performed FAS mediated apoptosis test. Eugenia Montanari, Michaela Calvillo, Filomena Pierri, Concetta Micalizzi, Rosario Maggiore, Daniela Guardo, Elena Facchini, Angela Maggio, Elena Mastrodicasa, Paola Corti, Giovanna Russo, Marta Pillon, Piero Farruggia, Simone Cesaro, Angelica Barone, Ugo Ramenghi: contributed essential data, cared for the patients. Jack Bleesing: discussed the results and critically reviewed the manuscript. Maurizio Miano, Carlo Dufour, Francesca Fioredda: designed the study protocol, contributed essential data, cared for the patients, revised the manuscript.

Data availability: the datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval: this study was performed in line with the principles of the Declaration of Helsinki.

Consent to participate: informed consent was obtained from the parents and/or individuals participants included in the study.

Consent to publish: not applicable.

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References

1. Bleesing et al, Völkl S, Rensing-Ehl A, Allgäuer A, et al. Hyperactive mTOR pathway promotes lymphoproliferation and abnormal differentiation in autoimmune lymphoproliferative syndrome. *Blood*. 2016;128(2):227–238. doi:10.1182/blood-2015-11-685024
2. Oliveira JB, Bleesing JJ, Dianzani U, Fleisher TA, Jaffe ES, Teachey DT, Rao VK. Et al. Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. *Blood*. 2010 Oct 7;116(14):e35-40. doi: 10.1182/blood-2010-04-280347.
3. Teachey, DT. New Advances in the Diagnosis and Treatment of Autoimmune Lymphoproliferative Syndrome (ALPS). *Current opinion in pediatrics* 24.1 (2012): 1.
4. Campagnoli, Maria Francesca, et al. The broad spectrum of autoimmune lymphoproliferative disease: molecular bases, clinical features and long-term follow-up in 31 patients. *Haematologica* 91.4 (2006): 538-541.
5. Sneller, Michael C., Janet K. Dale, and Stephen E. Straus. "Autoimmune lymphoproliferative syndrome." *Current opinion in rheumatology* 15.4 (2003): 417-421.
6. Bride K, Teachey DT. Autoimmune lymphoproliferative syndrome: more than a FAScinating disease. *F1000Research*. 2017;6, 1928
7. Bleesing JJ. Sorting out the causes of ALPS. *J Pediatr*. 2005;147(5):571–574.
8. Bleesing JJ, Brown MR, Dale JK, et al. TcR-alpha/beta(+) CD4(-)CD8(-) T cells in humans with the autoimmune lymphoproliferative syndrome express a novel CD45 isoform that is analogous to murine B220 and represents a marker of altered O-glycan biosynthesis. *ClinImmunol*. 2001;100(3):314–324.

9. Bleesing JJ, Janik JE, Fleisher TA. Common expression of an unusual CD45 isoform on T cells from patients with large granular lymphocyte leukaemia and autoimmune lymphoproliferative syndrome. *Br J Haematol*. 2003;120(1):93–96.
10. Bleesing JJ, Straus SE, Fleisher TA. Autoimmune lymphoproliferative syndrome. A human disorder of abnormal lymphocyte survival. *PediatrClin North Am*. 2000 Dec;47(6):1291-310. Review.
11. Bleesing JJ, Brown MR, Straus SE, et al. Immunophenotypic profiles in families with autoimmune lymphoproliferative syndrome. *Blood*. 2001;98(8):2466–2473.
12. Bride KL, Vincent T, Smith-Whitley K, Lambert MP, Bleesing JJ, Seif AE, Manno CS, Casper J, Grupp SA and Teachey D. Sirolimus is effective in relapsed/refractory autoimmune cytopenias: results of a prospective multi-institutional trial. 2015 *Blood*;127(1):17-28
13. Rao. VK Approaches to managing autoimmune cytopenias in novel immunological disorders with genetic underpinnings like autoimmune lymphoproliferative syndrome. *Frontiers in Pediatrics*, 2015
14. Rao VK, Oliveira JB. How I treat autoimmune lymphoproliferative. *Blood*. 2011 Nov 24;118(22):5741-51. doi: 10.1182/blood-2011-07-325217. Epub 2011 Sep 1. Review.
15. Miano M, Scalzone M, Perri K, Palmisani E et al. Mycophenolatemofetil and Sirolimus as second or further line treatment in children with chronic refractory primitive or secondary autoimmune cytopenias: a single centre experience. *Bjh*.2015, 171,247-253
16. Miano M, Ramenghi U, Russo G, Rubert L, Barone A, et al. Mycophenolatemofetil for the treatment of children with immune thrombocytopenia and Evans syndrome. A retrospective data review from the Italian association of paediatric haematology/oncology. *Bjh*.2016
17. Teachey, David T., Alix E. Seif, and Stephan A. Grupp. "Advances in the management and understanding of autoimmune lymphoproliferative syndrome (ALPS)." *British journal of haematology* 148.2 (2010): 205-216.
18. Rieux-Laucat F. The genetic peaks in the ALPS. *Current Opinion in Immunology* 2017, 49:79
19. Palmisani E, Miano M, et al. Clinical features and therapeutic challenges of cytopenias belonging to ALPS and ALPS-related (ARS) phenotype. *British Journal of Haematology*. 2019; 184(5); 861-864
20. Clementi R, Chiocchetti A, Cappellano G, Cerutti E, Ferretti M, Orilieri E, Dianzani I, Ferrarini M, Bregni M, Danesino C, Bozzi V, Putti MC, Cerutti F, Cometa A, Locatelli F, Maccario R, Ramenghi U, and Dianzani U. Variations of the perforin gene in patients with autoimmunity/lymphoproliferation and defective Fas function. *Blood*. 2006 Nov 1;108(9):3079-84. doi: 10.1182/blood-2006-02-001412. Epub 2006 May 23. PMID: 16720836
21. Miano M, Cappelli E, Pezzulla A, Venè R, Grossi A, Terranova P et al. FAS-mediated apoptosis impairment in patients with ALPS/ALPS-like phenotype carrying variants on CASP10 gene. *Br J Haematol*. 2019 Nov;187(4):502-508. doi: 10.1111/bjh.16098.
22. Rao VK, Dowdell KC, Dale JK, et al. Pyrimethamine treatment does not ameliorate lymphoproliferation or autoimmune disease in MRL/lpr-/- mice or in patients with autoimmune lymphoproliferative syndrome. *Am J Hematol*. 2007;82(12):1049–1055
23. Lenardo MJ, Oliveira JB, Zheng L et al. ALPS-ten lessons from an international workshop on a genetic disease of apoptosis. *Immunity*. 2010;32(3):291–295.
24. Bride KL, Vincent T, Smith-Whitley K, Lambert MP, Bleesing JJ, Seif AE et al. Sirolimus is effective in relapsed/refractory autoimmune cytopenias: results of a prospective multi-institutional trial. *Blood*. 2016 Jan 7;127(1):17-28. doi: 10.1182/blood-2015-07-657981.
25. Grossi A, Miano M, Lanciotti M, Fioredda F, Guardo D, Palmisani E, Terranova P, Santamaria G, Caroli F, Caorsi R, Volpi S, Gattorno M, Dufour C and Ceccherini I. Targeted NGS Yields Pleintful Ultra-rare Variants in Inborn Errors of Immunity Patients. *Genes* 2021, 12, 1299.
26. Hadjadj J, Aladjidi N, Fernandes H, Leverger G, Magérus-Chatinet A, Mazerolles F et al; members of the French Reference Center for Pediatric Autoimmune Cytopenia (CEREVANCE). Pediatric Evans syndrome is associated with a high frequency of potentially damaging variants in immune genes. *Blood*. 2019 Jul 4;134(1):9-21. doi: 10.1182/blood-2018-11-887141.
27. Cagdas D, OskayHalaçlı SO, Tan C, Lo B, Çetinkaya PG, Esenboğa S et al. A Spectrum of Clinical Findings from ALPS to CVID: Several Novel LRBA Defects *Journal of Clinical Immunology* 39, 726–738(2019)
28. Besnard C. Pediatric-onset Evans syndrome: Heterogeneous presentation and high frequency of monogenic disorders including LRBA and CTLA4 mutations. *J Clin Immunol*. 2018 Mar;188:52-57. doi: 10.1016/j.clim.2017.12.009.

Tables

Table 1. Genetic variants

Pt	gene	inheritance	protein	zigosity	GnomAD	Varsom	Type variant
1	FAS	AD	p.Glu218Lys	HET	-	LP	Missense
2	FAS	AD	p.Glu218Lys	HET	-	LP	Missense
3	FAS	AD	p.Glu194Lys/p.Ser225Thr	HET	1,41E-03	VUS/VUS	Missense/Missense
4	FAS	AD	p.Cys129Arg	HET	3.19E-5	LP	Missense
5	FAS	AD	p.Gly286Ter	HET	-	P	Nonsense
6	FAS	AD	p.Gln273His	HET	-	LP	Missense
7	FAS	AD	p.Gln273His	HET	-	LP	Missense
8	FAS	AD	p.Val190Met	HET	-	LP	Missense
9	FAS	AD	c.650_651+3delCTGTAAinsAGTG	HET		LP	frameshift
10	FAS	AD	p.Gly66Cys	HET		P	missense
11	FAS	AD	p.Val190Met	HET		LP	Missense
12	FAS	AD	c.650_651+3delCTGTAAinsAGTG	HET		LP	frameshift
13	CASP10	AD	p.Ile406Leu	HET	4,53E-02	LB	Missense
14	CASP10	AD	p.Ile406Leu	HET	4,53E-02	LB	Missense
15	CASP10	AD	p.Tyr446Cys	HET	2,28E-02	B	Missense
16	CASP10	AD	p.Val410Ile	HET	4.19E-02	B	Missense
17	CASP10	AD	p.Pro501Leu	HET	9,07E-04	B	Missense
18	CASP10	AD	p.Val410Ile	HET	4.19E-02	B	Missense
19	ARPC1B	AR		HOMO	4.22E-06	P	Missense
20	AIRE	AR/AD	p.Arg9Trp/p.Val484Met	HET	-	LP/VUS	Missense/Missense
21	STAT3	AD	p.Arg152Trp	HET	9,22E-05	LP	Missense
22	CTLA4/LRBA	AD/AR	p.Cys58Serfs*13/ p.Asp2294Asn	HET	-	LP/VUS	Frameshift/Missense
23	ADA2 STAT3	AR/AD	p.Leu188Pro-p.Thr187Pro7/ p.Lys658Arg	HET	-	LP-LP/LP	Missense-Missense/Missense
24	TNFRSF13C	AR	p.His159Tyr/p.Pro21Arg	HET	7.97E-06	B/B	Missense/Missense
25	NLRC4	AD	p.Arg492Trp	HET	1,97E-05	VUS	Missense
26	LRBA	AR	p.Gln2561Ter	HET	3,99E-06	P	Nonsense
27	LRBA	AR	p.Arg655Ter	HET	-	P	Missense
28	IKBKG	XLR	p.Glu125Lys	HET	5,81E-03	LP	Nonsense
29	TNFRSF13B	AD	p.Arg202His	HET	5,85E-02	VUS	Missense
30	GBA	AR	p.Asn370Ser/p.Gly202Arg	HET		P	missense
31	FOXP3	AD	p.Leu260Gln	HEMI		LP	missense
32	NEG						
33	NEG						
34	NEG						
35	NEG						
36	NEG						
37	NEG						
38	NEG						
39	NEG						
40	NEG						
41	NEG						
42	NEG						

43	NEG										
44	NEG										
45	NEG										
46	IKBKG	XLR	p.Glu125Lys		HET	1,50E-03	LP		Missense		

B, benign; LB, likely benign; P, pathogenic; LP likely pathogenic; VUS, variant of unknown significance

Table 2. Clinical and biochemical characteristic ALPS FAS/CASP10 patients

Pt n	Sex	Gene	Dg ALPS	DNT%>1,5%	Apoptosis	Lymphoproliferation	Cytopenia	Lymphocytopenia	IL- 10>20 pg/ml	IL- 18>500 pg/ml	VitB12>150 mg/dl
			Dg ALPS NIH criteria								
1	M	FAS	DEF	2,4	NP	Ly+S	N	N	NA	NA	NA
2	M	FAS	DEF	5,8	Pos	Ly+S	AIHA, ITP	N	35	550	291
3	M	FAS	DEF	6,1	Pos	S	AIHA	N	NA	NA	467
4	F	FAS	DEF	6,3	Pos	Ly+S	ITP, L	N	79	1175	2000
5	M	FAS	DEF	13	Pos	Ly+S	AIHA, ITP	N	NA	NA	1581
6	F	FAS	DEF	5,5	Neg	S	L, AIHA, ITP	Y	40	950	10233
7	M	FAS	DEF	3,7	Pos	Ly+S	N	N	0	1321	1747
8	M	FAS	DEF	4,8	Pos	Ly+S	ITP	N	NA	NA	879
9	M	FAS	DEF	18	Pos	Ly+S	ITP	N	NA	NA	2000
10	M	FAS	DEF	13	Neg	Ly+S	ITP+AIHA	N	NA	NA	1940
11	F	FAS	DEF	3,7	Pos	Ly	NA	NA	NA	NA	376
12	F	FAS	DEF	22	Pos	Ly+S	ITP+AIHA+L	N	115	1700	849
13	M	CASP10	DEF	6,3	Neg	Ly	NA	NA	0	567	NA
14	F	CASP10	DEF	1,9	Pos	S	ITP	N	0,9	250	547
15	F	CASP10	DEF	2,3	Neg	Ly+S	N	N	0	1050	393
16	F	CASP10	DEF	4,1	Pos	Ly+S	L, AIHA	N	90	5001	1492
17	M	CASP10	DEF	2,6	Pos	S	ITP	N	7,2	230	585
18	F	CASP10	DEF	3,1	Pos	Ly+S	ITP	N	3,1	1200	373

DEF, definitive; NP, not proliferative; Pos, pathologic; Neg, negative; Ly, lymphoproliferation; S, splenomegaly, NA, not applicable; Neg, negative; Y, present; AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenia; L, leucopenia; AIN, autoimmune neutropenia; DAT, direct antibodies test; ANA, anti nuclear antibodies; anti N, anti neutrophil antibodies; anti PLT, anti platelets antibodies; ASMA, anti smooth muscle antibodies

Table 2. Clinical and biochemical characteristic ALPS-U patients

Pt n	Sex	Gene	Dg ALPS NIH criteria	DNT%>1,5%	Apoptosis	Lymphoproliferation	Cytopenia	Lymphocytopenia	IL- 10>20 pg/ml	IL- 18>500 pg/ml	VitB1 mg/d
19	M	ARPC1B c.64+1G>C	DEF	3,6	Pos	Ly	ITP	Y	NA	NA	NA
20	F	AIRE p.Arg9Trp AIRE p.Val484Met	DEF	3,1	Pos	S	ITP	N	11,2	245	1016
21	M	STAT3 p.Arg152Trp	PROB	3,5	ND	S	ITP+L	Y	4	500	NA
22	F	CTLA4 p.Cys58fs LRBA p.Asp2294Asn	DEF	2,2	Pos	Ly+S	AIHA+ITP+L	Y	7	425	370
23	F	CECR1 p.Leu188Pro CECR1 p.Thr187Pro Somatic STAT3 p.Lys658Arg	DEF	5,1	Pos	Ly+S	AIHA+ITP	N	14	5250	374
24	M	TNFRSF13C p.His159Tyr TNFRSF13C p.Pro21Arg	PROB	4	Neg	Ly+S	ITP	Y	3	660	356
25	F	NLRC4 p.Arg492Trp	DEF	2,1	Pos	Ly+S	N	N	2,5	100	345
26	F	LRBA p.Gln2561Ter LRBA c.1359+1G>A	DEF	2,6	Pos	Ly+S	ITP+L	N	NA	NA	NA
27	M	LRBA p.Arg655Ter	DEF	2,2	Pos	Ly+S	N	N	7,5	815	990
28	M	IKBKG p.Glu125Lys	DEF	9	Pos	Ly+S	ITP	N	3	450	316
29	F	TACI p.Arg202His	DEF	5	Pos	Ly+S	ITP+AIHA+L	Y	0,9	1225	807
30	M	GBA p.Asn370Ser GBA p.Gly202Arg	DEF	2,8	Pos	Ly+S	ITP+L	Y	4	775	NA
31	M	FAS p.Ala16Thr	DEF	3,7	Neg	Ly	N	N	4	600	1176
32	F	NEG	PROB	2,5	Neg	S	L	N	11	475	1029
33	M	NEG	DEF	2	Pos	S	L	N	0,9	736	819
34	M	NEG	PROB	4,6	Neg	Ly+S	ITP+L	Y	2	600	274
35	M	NEG	PROB	2,2	Neg	Ly+S	ITP	N	2,4	900	529
36	F	NEG	PROB	2,4	Neg	Ly+S	N	N	0	700	1400
37	M	NEG	DEF	1,8	Pos	S	ITP+L	Y	305	360	725
38	M	NEG	PROB	2,5	Neg	Ly+S	L	N	0,9	925	567

39	M	NEG	DEF	1,7	Pos	S	ITP+L	N	3,5	2150	430
40	F	NEG	DEF	1,9	Pos	S	N	N	0	375	698
41	F	NEG	DEF	2,3	Pos	S	AIHA+ITP	Y	NA	NA	NA
42	F	NEG	DEF	2,4	Pos	Ly+S	ITP	N	12	1165	339
43	M	NEG	PROB	3,4	Neg	S	AIHA+L	Y	0	275	NA
44	M	NEG	PROB	3,5	Neg	Ly+S	ITP+L	N	13	600	641
45	M	NEG	DEF	14,8	Pos	Ly	AIHA+ITP+L	Y	0	550	684
46	F	IKBKG p.E125K XLR (Alice) LP	DEF	4,7	Pos	S	AIHA+ITP+L	Y	0,2	265	999

DEF, definitive; PROB, probable; NP, not proliferative; Pos, pathologic; Neg, negative; ND, not done; Ly, lymphoproliferation; S, splenomegaly, NA, not applicable; Neg, negative; Y, present; AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenia; L, leucopenia; AIN, autoimmune neutropenia; DAT, direct antibodies test; ANA, anti nuclear antibodies; anti N, anti neutrophil antibodies; anti PLT, anti platelets antibodies; ASMA, anti smooth muscle antibodies; CD, celiac disease; anti GP; anti gastric parietal cells; ICA, anti pancreatic insula

Table 3: statistical differences of clinical and biochemical characteristic and response to treatment

	ALPS FAS/CASP +	ALPS-U	P
Cytopenia(overall)	13/16 (81%)	23/28 (82%)	0.94
Trombocytopenia (ITP)	11/13 (85%)	19/23 (83%)	0.87
Haemolytic anemia (AIHA)	7/13 (54%)	7/23 (30%)	0.16
Autoimmune Neutropenia (AIN)	4/13 (31%)	15/23 (65%)	0.04
Lymphocytopenia	1/16 (6%)	16/28 (57%)	0.01
Isolated ITP	5/13 (38%)	6/23 (26%)	0.43
Isolated AIHA	1/13 (14%)	0/23 (0%)	0.17
Isolated leucopenia	0/13 (0%)	3/23 (13%)	0.17
Isolated AIN	0/13 (0%)	3/23 (13%)	0.17
ITP + AIHA	3/13 (23%)	2/23 (9%)	0.23
ITP+ leucopenia	1/13 (8%)	7/23 (30%)	0.11
AIHA + leucopenia	1/13 (8%)	1/23 (4%)	0.67
AIHA + leucopenia + ITP	2/13 (15%)	4/23 (17%)	0.87
Isolated lymphoproliferation	2/18 (11%)	3/28 (10%)	0.96
Isolated splenomegaly	4/18 (22%)	10/28 (36%)	0.33
Lymphoproliferation + splenomegaly	12/18 (67%)	15/28 (54%)	0.37
Vitamin B12 >1500ng/L	6/16 (37%)	0/22 (4%)	0.001
IL-10 >20pg/mL	5/11 (45%)	1/25 (4%)	0.002
IL-18 >500pg/mL	9/11 (82%)	16/25 (64%)	0.28
Autoimmunity markers	6/18 (33%)	19/28 (68%)	0.02
FAS-mediated Apoptosis	13/18 (72%)	18/27 (67%)	0.69
Multiorgan involvement	4/18 (22%)	21/28 (75%)	0.001
No therapy	2/18 (11%)	1/28 (4%)	0.31
Response to MMF or rapamycin*	12/12 (100%)	7/21 (33%)	0.0001
Associated therapies	1/16 (6%)	17/27 (63%)	0.0003
Number of drugs	2/16 (12%)	14/27 (52%)	0.009

Table 4 Flow cytometry as a diagnostic tools for ALPS screening

	ALPS FAS CASP +	ALPS-U	P
1. CD3CD4-CD8+TCRαβ+ (DN) (>1.5%)	18/18 (100%)	28/28 (100%)	
2. CD19+CD27+ (<15%)	8/11 (73%)	19/26 (73%)	0.98
3. TCR αβ+ B220+ (>60%)	9/11 (82%)	14/26 (50%)	0.10
4. CD3CD25+/CD3HLADR+ (<1)	9/11 (82%)	18/26 (69%)	0.43
Concomitant Pathological values A+B+C+D	7/11 (64%)	7/26 (27%)	0.01