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

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ABSTRACT

Autoimmune lymphoproliferative syndrome (ALPS) is an inherited disorder of lymphocyte homeostasis classically due to mutation of FAS, FASL, and CASP10 genes (ALPS-FAS/CASP10). Despite recent progress, about one-third of ALPS patients does not carry classical mutations and still remains gene orphan (ALPS-U, undetermined genetic defects). The aims of the present study were to compare the clinical and immunological features of ALPS-FAS/CASP10 versus those of ALPS-U affected subjects and to deepen the genetic characteristics of this latter group. Demographical, anamnestic, biochemical data were retrieved from medical record of 46 ALPS subjects. An enlarged panel of genes (next-generation sequencing) was applied to the ALPS-U group. ALPS-U subjects showed a more complex phenotype if compared to ALPS-FAS/CASP10 group, characterized by multiorgan 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 the 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 ALPS-FAS/CASP10 patients, while 63% of ALPS-U needed >2 lines of treatment and remission in some cases was obtained only after target therapy. In the ALPS-U group, we found in 14 of 28 (50%) patients 19 variants; of these, 4 of 19 (21%) were known as pathogenic and 8 of 19 (42%) as likely pathogenic. A characteristic flow cytometry panel including CD3CD4-CD8-+TCRαβ+, CD3+CD25+/CD3HLADR+, TCR αβ+ B220+, and CD19+CD27+ identified the ALPS-FAS/CASP10 group. ALPS-U seems to represent a distinct entity from ALPS-FAS/CASP10; this is relevant for management and tailored treatments whenever available.

INTRODUCTION

Autoimmune lymphoproliferative syndrome (ALPS) is a rare inherited disorder due to the dysregulation of the FAS apoptotic pathway, usually manifested in childhood, although symptoms may occur at any age. As a result of disturbed lymphocyte homeostasis, autoreactive cells persist, leading to the development of chronic nonmalignant lymphadenopathy, splenomegaly, multilineage cytopenias, and increased risk of lymphoma.[1] Currently, the diagnosis of ALPS is based on phenotypical criteria since it is determined by the combination of different clinical and laboratory findings as from reported in 2009

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The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.
This study was performed in line with the principles of the Declaration of Helsinki. Informed consent was obtained from the parents and/or individuals participants included in the study.

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by a panel of NIH (National Institutes of Health) 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) IL-10, IL-18, vitamin B12, and soluble FAS-ligand (sFASL).[6]

Elevated DNTs lymphocytes expressing B220,[7–9] 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-+TCRαβ+, CD3+CD25+/CD3HLA DR+, TCRαβ+ B220+, and CD19+CD27+.

Management of ALPS patients aims to control clinical manifestations and specific complications such as cytopenia are often difficult to handle because stable remission may be difficult to achieve after the first (steroids and immunoglobulins) and even the second line of treatment (mychopenolate-mophetil [MMF] and rapamycin).[12–16]

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 2 groups ALPS-FAS/CASP10 versus ALPS-U to identify any possible surrogate markers that may direct the diagnosis toward more precise genetic categories.

Table 1

ALPS Diagnostic Criteria: Report From the 2009 NIH International Workshop

Required
1. Chronic nonmalignant lymphoproliferation (<6 mo lymphadenopathy and/or splenomegaly)
2. Elevated peripheral blood DNTs
Accessory
Primary
1. Defective in vitro Fas-mediated apoptosis (in 2 separate assays)
2. Somatic or germline mutation in ALPS causative gene (FAS, FASL, CASP10)
Secondary
1. Elevated biomarkers (any of the following): (a) Plasma sFASL > 200 pg/mL; (b) Plasma IL-10 > 20 pg/mL; (c) Plasma or serum vitamin B12 > 1500 ng/L; (d) Plasma IL-18 > 500 pg/mL
2. Immunohistochemical findings consistent with ALPS as determined by experienced histopathologist
3. Autoimmune cytopenias AND polyclonal hypergammaglobulinemia
4. Family history of ALPS or nonmalignant lymphoproliferation
Definitive diagnosis: the presence of both required criteria plus one primary accessory criterion.
Probable diagnosis: both required criteria plus one secondary accessory criterion.
Of note, probable and definitive ALPS should be treated the same in the clinic.

ALPS = autoimmune lymphoproliferative syndrome; DNTs = double-negative T cells; NIH = National Institutes of Health; sFASL = soluble FAS-ligand.

PATIENTS AND METHODS

Patients

Patients were defined as ALPS definitive and probable according to the criteria established by the NIH in 2009.[2] (Table 1) 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 alfabeta+, named DNT cells), autoimmune markers (antiplatelet and antineutrophil indirect antibodies, direct and indirect Coombs test, antinuclear antibodies [ANA], extractable nuclear antigen antibodies, antismooth muscle antibody [ASMA], anti double-stranded DNA [anti-dsDNA] antibodies, antiglyadin, antitransglutaminase, antiendomysium, and antithyroglobulin antibodies) were entered into an electronic national ALPS database (ALPS Italian Network) (Tables 4 and 5).

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 nonresponse (NR) (See for further detail the footnotes of Table 6). 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 nonresponder if none of the above symptoms improved/normalized 4–6 weeks after treatment start.

First-line treatment was steroids or intravenous immunoglobulins, whereas the second-line was 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 4 parameters suggestive of ALPS (CD3CD4-CD8+TCRαβ+>1.5% of total lymphocytes, CD3CD25+/CD3HLADR+<1%, TCR αβ+ 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 8-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).

Functional tests

FAS-mediated apoptosis test

Fas-induced cell death was evaluated on T-cell lines obtained by activating peripheral blood mononuclear cells (PBMCs) with phytohemagglutinin (PHA) at days 0 (1 μg/mL) and 12–15 (0.1 μg/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 μg/mL) in the presence of rIL-2 (1 U/mL) to minimize spontaneous cell death. Cell survival was evaluated after 18 hours by counting live cells in each well by the trypan blue exclusion test and by flow cytometry of cells excluding propidium iodide. Cells from 2 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) × 100. Fas function was defined as defective when cell survival was >78% (the 95th percentile of data obtained from 200 normal controls).[20]

Not proliferative (NP) indicates that tested lymphocyte are normally culture for 18 days and occasionally at time of testing cells may not proliferate in sufficient number. The cells are kept in culture for 18 days to select the population of T-lymphocytes on which to perform the test. In some cases it may happen that at the time of setting up the test the number of cells is insufficient, and in this case, we indicate insufficient cell proliferation in the report.

ADA2 functional test

Two previously unreported variants of gene ADA2 were found through next-generation sequencing (NGS) analysis, and a pathogenic role was demonstrated through a functional study.

A functional analysis on peripheral monocytes was performed to test their effect on ADA2 activity.

These cells were isolated by adherence, after PBMC Ficoll–Paque separation and were then cultured in phosphate-buffered saline with exogenous adenosine (Sigma Aldrich) with or without ADA1 inhibitorerythro-9-(2-hydroxy-3-nonyl) adenine (Sigma Aldrich) for4 h at 37°C with 5% of CO_2. The supernatants were collected, and the activity enzyme was indirectly evaluated in high-performance liquid chromatography through the measurement of the adenosine-derived products (inosine and hypoxanthine) as a surrogate marker of enzyme activity.[21]

CASP10 functional test

In CASP10 variants, functional studies were performed after inducing apoptosis by FAS-ligand/TRIAL stimulation and analyzing cell death and the function of CASP10, CASP8 and PARP proteins. Human lymphoblast cell lines, obtained from patients' samples after informed consent, were grown in RPMI medium with glutamine and antibiotics to 37°C and 5% CO_2. Recombinant FAS-ligand (FASL) was from Enzo Life Science (Farmingdale, NY) and recombinant super killer TRAIL was from Alexis Biochemicals (Farmingdale). Both primary cells and lymphoblasts were treated with FASL(10 ng/mL) or TRAIL (100 ng/mL) for 24 hours to induce apoptosis and cell death, which were measured by the cytofluorimeter. For Western blotting, the cells were incubated for 4 hours, then lysed with ice-cold radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. Protein quantification in cell lysates was done with the DC Protein assay Kit (BioRad, Hercules, CA). An equal amount of protein (20 µg/lane) were loaded on precast 8% gel (GenScript, Piscataway, NJ) and electrotransferred to polyvinylidene difluoride membranes (GE Healthcare, Chicago, IL). After blocking, membranes were probed over night at 4°C with the following antihuman primary antibodies diluted according to the manufacturer's instructions: mouse monoclonal anti-Caspase-8 (1C12), rabbit polyclonal anti-PARP (Cell Signaling, Danvers, MA) and rabbit monoclonal anti-Caspase-10 (Abcam, Cambridge, United Kingdom). After washing, the membranes were incubated for 1 hours at room temperature with the relevant horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling) and proteins were detected by chemiluminescent HRP substrate (Immobilon Western, Millipore, Burlington, MA). Anti-actin HRP-conjugated (Cell Signaling) was used as loading control. To better investigate the causes of this resistance to apoptosis, we assessed cell extracts after treatment with FAS-L and TRAIL by Western blotting.

Interleukin 18, Interleukin 10, and FasL

Serum interleukin 18, interleukin 10, and FasL assays were performed using commercially available enzyme-linked immunosorbent assay kits (MBL, Woburn, MA; Invitrogen, Waltham, MA; and Abnova, Taipei, Taiwan, respectively).

Genetic analysis

Forthysix 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 of 18 subjects of the ALPS-FAS/CASP10 group and 3 of 28 of the ALPS-U group or by NGS with Sanger confirmation (the most recent diagnoses; 8/18 patients of the ALPS-FAS/CASP10 group and 25/28 of the ALPS-U group).

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

We used the Sanger PCR technique to search for mutations in the pre-NGS period. We studied with NGS new patients who had not yet undergone any molecular investigation. To confirm the presence of the variants thus selected, the Sanger PCR protocol was set up for each variant to confirm genetic mutations detected by NGS.

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 hematological disorders, immunodeficiencies, immune dysregulation, inflammatory, and bone marrow failure syndromes (Table 2).

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, the 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 Epstein-Barr virus-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 the fresh blood.

Statistical analysis

Descriptive statistics were reported in terms of absolute frequencies and percentages. Distribution of data regarding continuous variables was described in terms of a median value and range. Comparison of frequency distribution was analyzed by the Chi-square test. The Fisher exact test was used in the case of at least one expected frequency <5. Comparison regarding demographic characteristics and double-negative values was analyzed by the Mann–Whitney test as nonparametric test. All test were two-tailed and *P* value <0.05 was considered statistically significant.

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

RESULTS

Characteristic of the whole cohort

Forty-six 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. In the ALPS-FAS/CASP10 group, all patients were affected with definitive ALPS; in the ALPS-U group, 19 of 28 were definitive.

Thirty-seven of 46 (81%) were affected with definitive ALPS and 9 of 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 1–39 years). Median duration of follow-up of the whole cohort was

Table 2

List of Genes Included in Panel

Target ID	Coverage	Target ID	Coverage	Target ID	Coverage	Target ID	Coverage	Target ID	Coverage
A20	99.49	CENPS	100	HAX1	100	NHEJ1	100	SERPING1	100
ACP5	100	CENPX	100	HOIP	100	NLRC4	100	SH2D1A	100
ACT1	100	CFB	100	ICOS	100	NLRP12	100	SH3BP2	100
ACTB	61.77	CFD	100	IFIH1	100	NLRP3	100	SLC29A3	100
ADA2	100	CFH	100	IFNGR1	100	NLRP7	99.77	SLC37A4	100
ADAR1	100	CFHR1	95.24	IFNGR2	100	NOD2	100	SLC46A1	100
AICDA	100	CFHR3	88.54	IGLL1	100	NOLA2	100	SLC7A7	100
AIRE	100	CFI	100	IKAROS	100	NOLA3	100	SLX4	100
AK2	100	CFP	100	IKBA	100	NRAS	100	SMARCAL1	100
AP1S3	100	CHD7	100	IKBKB	100	ORAI1	100	SP110	100
AP3B1	100	CIITA	100	IKBKG	46.03	OTULIN	100	SPINK5	100
APOL1	100	COLEC11	100	IKZF1	100	OX40	100	STAT1	100
ARPC1B	100	COPA	100	IL-10	100	PALB2	100	STAT2	100
ATM	100	CORO1A	95.62	IL10RA	100	PAX5	100	STAT3	100
BCL10	100	CSF2RA	22.43	IL10RB	100	PGM3	100	STAT5B	90.23
BLM	100	CSF3R	100	IL12B	100	PI3K	99.63	STIM1	100
BLNK	99.46	CTC1	100	IL12RB1	100	PIK3CD	100	STK4	100
BLOC1S6	100	CTLA4	100	IL17F	100	PIK3R1	100	STN1	100
BOD1L1	100	CTPS1	100	IL17RA	100	PLCG2	100	STX11	100
BRCA1	100	CTSC	100	IL1RN	100	PMS2	74.9	STXBP2	98.89
BRCA2	99.97	CXCR4	100	IL21	100	PNP	100	TAP1	100
BRIP1	100	CYBA	100	IL21R	100	POLE1	99.89	TAP2	99.85
BTK	100	CYBB	100	IL2RA	100	PRF1	100	TAPBP	100
C1NH	100	DCLRE1C	100	IL2RG	100	PRKCD	100	TAZ	100
C1QA	100	DKC1	100	IL36RN	100	PSMA3	100	TBK1	100
C1QB	100	DNASE1	100	IL7R	100	PSMB4	100	TBX1	100
C1QC	100	DNASE1L3	100	IRAK4	100	PSMB8	100	TCF3	100
C1R	100	DNASE2	100	IRF8	100	PSMB9	100	TCN2	100
C1S	100	DNMT3B	100	ISG15	100	PSTPIP1	99.53	TERC	100
C2	99.06	DOCK2	100	ITCH	100	PTPRC	97.54	TERT	100
C3	100	DOCK8	100	ITGB2	100	RAB27A	100	THBD	100
C4A	21.47	ELANE	100	ITK	100	RAC2	100	TINF2	100
C4B	27.75	ERCC4	100	JAGN1	100	RAD51	100	TLR3	100
C5	100	EVER1	100	JAK1	100	RAD51C	100	TMEM173	100
C6	100	EVER2	100	JAK3	100	RAG1	100	TNFAIP3	100
C7	100	EXTL3	100	KIND3	100	RAG2	100	TNFRSF11A	100
C8A	100	FAAP100	100	KRAS	100	RASGRP1	100	TNFRSF13B	100
C8B	100	FAAP20	100	LACC1	100	RBCK1	100	TNFRSF13C	100
C8G	100	FAAP24	100	LAMTOR2	100	RFX5	100	TNFRSF1A	98.34
C9	96.03	FADD	100	LCK	100	RFXANK	93.94	TPP2	100
CARD11	100	FAN1	100	LIG4	100	RFXAP	100	TRAF3	100
CARD14	100	FANCA	97.66	LPIN2	100	RHOH	100	TREX1	100
CARD9	100	FANCB	100	LRBA	99.94	RMRP	100	TRIF	100
CASP10	100	FANCC	100	LYST	99.99	RNASEH2A	100	TTC7A	100
CASP8	100	FANCD2	99.42	MAGT1	99.4	RNASEH2B	100	TWEAK	100
CD19	100	FANCE	100	MALT1	100	RNASEH2C	100	TYK2	100
CD20	99.57	FANCF	100	MAP3K14	100	RNF168	100	UAF1	100
CD21	100	FANCG	100	MASP1	100	RPL11	100	UBE2T	100
CD27	100	FANCI	100	MASP2	100	RPL26	100	UNC119	100
CD3D	100	FANCL	100	MCM4	100	RPL35A	100	UNC13D	100
CD3E	100	FANCM	100	MDA5	100	RPL5	100	UNC93B1	95.43
CD3G	99.65	FAS	100	MEFV	100	RPS10	100	UNG	100
CD3Z	100	FASLG	100	MPL	100	RPS17	15.97	USB1	100
CD40	100	FCN3	100	MRE11	100	RPS19	100	USP1	100
CD40LG	100	FOXN1	100	MTHFD1	100	RPS24	100	VPS13B	100
CD46	100	FOXP3	100	MVK	100	RPS26	100	VPS45	100
CD59	100	FPR1	100	MYD88	100	RPS7	100	WAS	100
CD70	99.52	FUCT1	100	NBN	100	RPSA	100	WDR1	100
CD79A	100	G6PC	100	NCF1	42.96	RTEL1	100	WIPF1	100
CD79B	100	G6PC3	100	NCF2	100	RUNX1	99.94	WRAP53	100
CD81	100	GATA2	100	NCF4	100	SAMHD1	100	XIAP	100
CD8A	100	GFI1	100	NFKB2	100	SBDS	92.88	ZAP70	100
CEBPE	100	GIMAP5	100	NFKBID	100	SEMA3E	100	ZBTB24	100

3.3 years (range 0–14 years). Patients were genetically tested 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 cytometry markers and 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 3).

Patient 1 and patient 2 (Table 3) are brothers. Both have lymphoproliferation, increased DNTs and one of them pathological FAS assay, while in the other the test failed. Moreover, one of them needs therapy for immune thrombocytopenia. The Varsome data base considers their variants as likely pathogenic. Putting together the biochemical and the immunological data, we reckon that these patients can be considered as having ALPS disease.

In patient 3 (Table 3), the variant is uncertain, but it was associated to a pathological FAS test and to clinical findings very suggestive of ALPS including the presence of lymphoproliferation, increased DNTs, and autoimmune cytopenia (AIHA).

Table 3

Genetic Variants

Pt	Gene	Inheritance	Protein	Zigosity	Segregation	GnomAD	Varsom	Type Variant
1	FAS	AD	p.Glu218Lys	HET	na	–	LP	Missense
2	FAS	AD	p.Glu218Lys	HET	na	–	LP	Missense
3	FAS	AD	p.Glu194Lys/p.Ser225Thr	HET	na	1.41E-03	VUS/VUS	Missense/Missense
4	FAS	AD	p.Cys129Arg	HET	Mother	3.19E-5	LP	Missense
5	FAS	AD	p.Gly286Ter	HET	na	–	P	Nonsense
6	FAS	AD	p.Gln273His	HET	na	–	LP	Missense
7	FAS	AD	p.Gln273His	HET	na	–	LP	Missense
8	FAS	AD	p.Val190Met	HET	na	–	LP	Missense
9	FAS	AD	c.650_651 + 3delCTGTAinsAGTG	HET	na		LP	Frameshift
10	FAS	AD	p.Gly66Cys	HET	na		P	Missense
11	FAS	AD	p.Val190Met	HET	na		LP	Missense
12	FAS	AD	c.650_651 + 3delCTGTAinsAGTG	HET	na		LP	Frameshift
13	CASP10	AD	p.Ile406Leu	HET	na	4.53E-02	LB	Missense
14	CASP10	AD	p.Ile406Leu	HET	Father	4.53E-02	LB	Missense
15	CASP10	AD	p.Tyr446Cys	HET	Mother	2.28E-02	B	Missense
16	CASP10	AD	p.Val410Ile	HET	Mother	4.19E-02	B	Missense
17	CASP10	AD	p.Pro501Leu	HET	Mother	9.07E-04	B	Missense
18	CASP10	AD	p.Val410Ile	HET	na	4.19E-02	B	Missense
19	ARPC1B	AR		HOMO	na	4.22E-06	P	Missense
20	AIRE	AR/AD	p.Arg9Trp/p.Val484Met	HET	na	–	LP/VUS	Missense/Missense
21	STAT3	AD	p.Arg152Trp	HET	na	9.22E-05	LP	Missense
22	CTLA4/LRBA	AD/AR	p.Cys58Serfs*13/p.Asp2294Asn	HET	Mother/mother	–	LP/VUS	Frameshift/Missense
23	ADA2 STAT3	AR/AD	p.Leu188Pro-p.Thr187Pro7/p.Lys658Arg	HET	Father/somatic	–	LP-LP/LP	Missense-Missense/Missense
24	TNFRSF13C	AR	p.His159Tyr/p.Pro21Arg	HET	Father	7.97E-06	B/B	Missense/Missense
25	NLRC4	AD	p.Arg492Trp	HET	na	1.97E-05	VUS	Missense
26	LRBA	AR	p.Gln2561Ter	HET	na	3.99E-06	P	Nonsense
27	LRBA	AR	p.Arg655Ter	HET	Mother/father	–	P	Missense
28	IKBKG	XLR	p.Glu125Lys	HET	Mother	5.81E-03	LP	Nonsense
29	TNFRSF13B	AD	p.Arg202His	HET	Father	5.85E-02	VUS	Missense
30	GBA	AR	p.Asn370Ser/p.Gly202Arg	HET	na		P	Missense
31	FOXP3	AD	p.Leu260Gln	HEMI	na		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	mother	1.50E-03	LP	Missense

AD = autosomal dominant; AR = autosomal recessive; B = benign; HEMI = hemizygous; HET = heterozygous; HOMO = homozygous; LB = likely benign; LP = likely pathogenic; na = not available; NEG = negative; P = pathogenic; VUS = variant of unknown significance; XLR = X-linked-related.

According to Varsome, the variant p.Cys129Arg is likely pathogenic. Indeed, in patient 4 (Table 3), there are other elements supporting the pathogenic role of this variant including lymphoproliferation increase of DNTs and pathological FAS test.

Patients 6 and 7 (Table 3) with p.Gln273His variant are related (father and daughter).

According to Varsome, this variant is likely pathogenic. These patients have lymphoproliferation, increase of DNTs, pathological FAS test (only patient 7) AIHA, ITP, and leucopenia (only patient 7).

The 2 CASP10 p.Tyr446Cys and CASP10 p.Val410Ile variants found in patients 15, 16, and 18, respectively, known as benign were found to be associated with impaired apoptosis driven by FAS-ligand and TRAIL stimulation[22] (Table 1). The remaining 4 variants are considered variant of unknown significance (VUS) or likely benign or benign.

Within the ALPS-U group, we found in 14 of 28 (50%) patients 19 variants; of these, 4 of 19 (21%) were known as pathogenic, 8 of 19 (42%) as likely pathogenic and 4 of 19 (21%) as VUS. In 1 patient (ALPS-U Pt 23), we found 2 ADA2 gene variants, reported as VUS/likely pathogenic, which was associated to reduced expression (assessed in Western blot) of the corresponding ADA2 protein (Table 3).

We outline that on the clinical point of view, some VUS may be of relevance if associated to a clinical phenotype that is clearly consistent with the diagnosis. In the specific case of patient 24 (Table 3), we reckoned that these 2 VUS in the context of the clinical phenotype might have been somehow contributory.[23]

Patient 23 (Table 3) showed 3 different causative variants. One rare STAT3 variant was a somatic mosaicism, being present in the DNA extracted from peripheral blood but not in the DNA extracted from a different source. Two other rare variants affected the 2 different alleles of the ADA2 gene: indeed, the parents turned out to carry one variant each (p.Leu188Pro for the mother and p.Thr187Pro for the father).[24–26]

In patient 27, genetic analysis detected a pathogenic heterozygous variant of LRBA. This patient has a composite clinical phenotype including symptoms fully compatible with a diagnosis of ALPS but also others not consistent with this diagnosis (diarrhea, recurrent upper respiratory tract infections).[27,28]

Patients 28 carried pathogenic variants in the X-linked IKBKG gene, showing a clinical overlap between inborn errors of immunity and bone marrow failure, in line with what was recently demonstrated.[25]

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 6).

As expected, chronic (<6 months) nonmalignant lymphoproliferation expressed by lymphadenopathy and/or splenomegaly[14,29] was present in all patients in both groups. Lymphoadenopathy and splenomegaly was contemporarily present in 67% of the ALPS-FAS/CASP10 group and in 54% of the ALPS-U group ($P = 0.37$).

Cytopenia affecting one or more hematopoietic lineages without difference in distribution between ALPS-FAS/CASP10 and ALPS-U (81% versus 82%; P = ns) groups.

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

A flow cytometry panel identifying 4 specific lymphocyte subpopulations (CD3CD4-CD8-+TCRαβ+, CD3+CD25+/CD3HLADR+, TCR αβ+ B220+, and CD19+CD27+) and proposed as a diagnostic tool for screening ALPS[30] was compared in ALPS-FAS/CASP10 versus ALPS-U. When taken individually, each of these lymphocyte subpopulation was not significantly different in the 2 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 7).

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 IL-10 (> 20pg/mL) ($P = 0.002$) (Table 6).

Not surprisingly, DNTs, 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%]; $P = 0.11$).

Somatic Fas mutations were not detected in our patient. This may reflect a lack of sensitivity of the sequencing assay because genetic analysis was not performed on sorted DNT cells.

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

Overall, markers of autoimmunity were positive in 6 of 18 (33%) subjects of the ALPS-FAS/CASP10 group and in 19 of 28 (68%) ALPS-U subjects ($P = 0.02$). In terms of frequency, direct antibodies test (DAT+) was the most common positive marker in the ALPS-FAS/CASP10 group (4/14; 28%), while ANAs were so in the ALPS-U group (7/28; 25%).

Signs and symptoms of autoimmune diseases (arthritis, celiac 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%) ($P = 0.001$).

Only the ALPS-U group comprised both definitive ALPS and probable ALPS and the only statistical differences between them was in FAS-mediated apoptosis ($P < 0.05$).

Treatment

Three 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 of 38 subjects (30%), 6of 13 (46%) in ALPS-FAS/CASP10, and 7 of 22 (32%) in the 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 the ALPS-U group ($P < 0.001$). 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 2 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 the ALPS-U group, a CR was achieved in 12 of 27 (44%) and a PR in 10 of 27 (37%).

Overall, ALPS-U received a significantly higher number of combination treatments (no 17/27, 63%) over ALPS-FAS/CAS10 (no 1/16.6%) ($P = 0.01$) and a significantly greater number of drugs (14/27 [52%] versus 2/16 [12%] of ALPS-FAS/CASP10) to achieve a response ($P = 0.009$) (Table 6).

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] (Table 1). 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 an increased frequency of lymphocytopenia, of associated autoimmune makers/symptoms and consequently

Table 4

Clinical and Biochemical Characteristic ALPS-FAS/CASP10 Patients

Pt n	Sex	Gene	Dg ALPS NIH criteria	DNT% >1,5%	Apoptosis	Lymphop-roliferation	Cytopenia	Lympho-cytopenia	IL-10 > 20 pg/mL	IL-18 > 500 pg/mL	VitB12 > 1500 mg/dL	CD19 +CD27 +<15%	TCR αβ B220+>60%	CD3CD25+/ CD3HLADR+<1	AUTOIMMUNE markers	Multiorgan Involvement
1	M	FAS	DEF	2.4	NP	Ly+S	Neg	Neg	NA	NA	NA	NA	NA	NA	Neg	Y
2	M	FAS	DEF	5.8	Pos	Ly+S	AIHA, ITP	Neg	35	550	291	12	89.8	0.5	DAT	Neg
3	M	FAS	DEF	6.1	Pos	S	AIHA	Neg	NA	NA	467	NA	NA	NA	Neg	Neg
4	F	FAS	DEF	6.3	Pos	Ly+S	ITP, L	Neg	79	1175	2000	10.3	86	0.3	Neg	Y
5	M	FAS	DEF	13	Pos	Ly+S	AIHA, ITP	Neg	NA	NA	1581	NA	NA	NA	DAT	Neg
6	F	FAS	DEF	5.5	Neg	S	L, AIHA, ITP	Y	40	950	10233	2.7	68.7	0.4	Neg	Neg
7	M	FAS	DEF	3.7	Pos	Ly+S	Neg	Neg	0	1321	1747	2.2	69.4	0.2	Neg	Neg
8	M	FAS	DEF	4.8	Pos	Ly+S	ITP	Neg	NA	NA	879	NA	NA	NA	Neg	Neg
9	M	FAS	DEF	18	Pos	Ly+S	ITP	Neg	NA	NA	2000	NA	NA	NA	Neg	Neg
10	M	FAS	DEF	13	Neg	Ly+S	ITP+AIHA	Neg	NA	NA	1940	NA	NA	NA	Neg	Neg
11	F	FAS	DEF	3.7	Pos	Ly	NA	NA	NA	NA	376	NA	NA	NA	Neg	Neg
12	F	FAS	DEF	22	Pos	Ly+S	ITP+AHIA+L	Neg	115	1700	849	4.6	89	0.1	Neg	Neg
13	M	CASP10	DEF	6.3	Neg	Ly	NA	NA	0	567	NA	25.7	66.4	1.2	Neg	Neg
14	F	CASP10	DEF	1.9	Pos	S	ITP	Neg	0.9	250	547	49	41.7	1.9	ANA	Neg
15	F	CASP10	DEF	2.3	Neg	Ly+S	Neg	Neg	0	1050	393	15	81	0.2	DAT	Neg
16	F	CASP10	DEF	4.1	Pos	Ly+S	L, AIHA	Neg	90	5001	1492	3	62.8	0.3	DAT, ASMA, anti N and PLT	Y
17	M	CASP10	DEF	2.6	Pos	S	ITP	Neg	7.2	230	585	13.6	10.4	0.3	Neg	Neg
18	F	CASP10	DEF	3.1	Pos	Ly+S	ITP	Neg	3.1	1200	373	18	73.5	0.2	ANA	Y

AIHA = autoimmune hemolytic anemia; AIN = autoimmune neutropenia; ALPS = autoimmune lymphoproliferative syndrome; ANA = antinuclear antibodies; antiN = antineutrophil antibodies; antiPLT = antiplatelets antibodies; ASMA = antismooth muscle antibodies; DAT = direct antibodies test; DEF = definitive; F = female; ITP = immune thrombocytopenia; L = leucopenia; Ly = lymphoproliferation; M = male; NA = not applicable; Neg = negative; NP = notproliferative; Pt = patient; Pos = pathologic; S = splenomegaly; Y = present.

Table 5

Clinical and Biochemical Characteristic ALPS-U Patients

Pt n	Sex	Gene	Dg ALPS NIH criteria	DNT% >1,5%	Apop-tosis	Lymphopro-liferation	Cytopenia	Lympho-cytopenia	IL-10 > 20 pg/mL	IL-18 > 500 pg/mL	VitB12 > 1500 mg/dL	CD19 +CD27 +<15%	TCR αβ B220+ >60%	CD3CD25+/ CD3HLADR+<1	Autoimmune Markers	Multiorgan Involvement
19	M	ARPC1B c.64 + 1G>C	DEF	3.6	Pos	Ly	ITP	Y	NA	NA	NA	10.6	64.7	0.8	ANA, ASCA	Y
20	F	AIRE p.Arg9Trp AIRE p.Val484Met	DEF	3.1	Pos	S	ITP	Neg	11.2	245	1016	9.4	29	1	Neg	Neg
21	M	STAT3 p.Arg152Trp	PROB	3.5	ND	S	ITP+L	Y	4	500	NA	14.7	48	0.8	CD	Y
22	F	CTLA4 p.Cys58fs LRBA p.Asp2294Asn	DEF	2.2	Pos	Ly+S	AIHA+ITP+L	Y	7	425	370	3.3	62.3	1.9	Anti GP	Y
23	F	ADA2 p.Leu188Pro ADA2 p.Thr187Pro Somatic STAT3 p.Lys658Arg	DEF	5.1	Pos	Ly+S	AIHA+ITP	Neg	14	5250	374	15.9	82.7	0	DAT, ANA	Y
24	M	TNFRSF13C p.His159Tyr TNFRSF13C p.Pro21Arg	PROB	4	Neg	Ly+S	ITP	Y	3	660	356	6.6	70.6	0.2	DAT	Neg
25	F	NLRC4 p.Arg492Trp	DEF	2.1	Pos	Ly+S	Neg	Neg	2.5	100	345	11	29.9	1.7	Neg	Y
26	F	LRBA p.Gln2561Ter LRBA c.1359 + 1G>A	DEF	2.6	Pos	Ly+S	ITP+L	Neg	NA	NA	NA	NA	NA	NA	DAT,ASMA,ICA,CD	Y
27	M	LRBA p.Arg655Ter	DEF	2.2	Pos	Ly+S	Neg	Neg	7.5	815	990	12.3	85	0.8	ANCA, ASMA	Y
28	M	IKBKG p.Glu125Lys	DEF	9	Pos	Ly+S	ITP	Neg	3	450	316	6.8	67.5	1	ANA, anti N	Y
29	F	TNFRSF13B p.Arg202His	DEF	5	Pos	Ly+S	ITP+AIHA+L	Y	0.9	1225	807	15	46.6	2.9	ANA, DAT, CD	Y
30	M	GBA p.Asn370Ser GBA p.Gly202Arg	DEF	2.8	Pos	Ly+S	ITP+L	Y	4	775	NA	9.4	56	1.5	Neg	Neg
31	M	FOXP3 p.Leu260Gln	DEF	3.7	Neg	Ly	Neg	Neg	4	600	1176	4.2	52	0.9	Neg	Neg
32	F	NEG	PROB	2.5	Neg	S	L	Neg	11	475	1029	22.3	62.5	1.3	ANA, ASCA, anti N	Y
33	M	NEG	DEF	2	Pos	S	L	Neg	0.9	736	819	14.4	61	3	Neg	Y
34	M	NEG	PROB	4.6	Neg	Ly+S	ITP+L	Y	2	600	274	6.2	70	0.9	ENA	Y
35	M	NEG	PROB	2.2	Neg	Ly+S	ITP	Neg	2.4	900	529	7.9	47.8	1.6	Neg	Y
36	F	NEG	PROB	2.4	Neg	Ly+S	Neg	Neg	0	700	1400	11.3	60	0.4	Neg	Y
37	M	NEG	DEF	1.8	Pos	S	ITP+L	Y	305	360	725	12.5	68.7	0.1	ANA,ENA,AS-MA,CD, anti N	Y
38	M	NEG	PROB	2.5	Neg	Ly+S	L	Neg	0.9	925	567	3.8	47.7	1	Neg	Y
39	M	NEG	DEF	1.7	Pos	S	ITP+L	Neg	3.5	2150	430	33.6	78.7	0.6	Anti N	Y
40	F	NEG	DEF	1.9	Pos	S	Neg	Neg	0	375	698	26.6	83	0.7	CD	Neg
41	F	NEG	DEF	2.3	Pos	S	AIHA+ITP	Y	NA	NA	NA	NA	NA	NA	DAT	Y
42	F	NEG	DEF	2.4	Pos	Ly+S	ITP	Neg	12	1165	339	13.9	57.4	0.3	Neg	Y
43	M	NEG	PROB	3.4	Neg	S	AIHA+L	Y	0	275	NA	23	48.3	0.8	ASMA, ASCA	Y
44	M	NEG	PROB	3.5	Neg	Ly+S	ITP+L	Neg	13	600	641	12.9	43.9	0.8	DAT	Neg
45	M	NEG	DEF	14.8	Pos	Ly	AIHA+ITP+L	Y	0	550	684	34.2	69	1	ANA,DAT	Neg
46	F	IKBKG p.Glu125Lys	DEF	4.7	Pos	S	AIHA+ITP+L	Y	0.2	265	999	0	59	2.4	ANA, CD, tyroid	Y

AIHA = autoimmune hemolytic anemia; AIN = autoimmune neutropenia; ALPS = autoimmune lymphoproliferative syndrome; ANA = antinuclear antibodies; antiGP = antigastric parietal cells; antiN = antineutrophil antibodies; antiPLT = antiplatelets antibodies; ASMA = antismooth muscle antibodies; CD = celiac disease; DAT = direct antibodies test; DEF = definitive; ICA = antipancreatic insula; F = female; ITP = immune thrombocytopenia; L = leucopenia; Ly = lymphoproliferation; M = male; NA = not applicable; Neg = negative; NP = not proliferative; Pos = pathologic ; Pt = patient; S = splenomegaly; Y = present.

Table 6

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
Thrombocytopenia (ITP)	11/13 (85%)	19/23 (83%)	0.87
Hemolytic 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 > 1500 ng/L	6/16 (37%)	0/22 (4%)	0.001
IL-10 > 20 pg/mL	5/11 (45%)	1/25 (4%)	0.002
IL-18 > 500 pg/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[a]	12/12 (100%)	7/21 (33%)	<0.001
Associated therapies	1/16 (6%)	17/27 (63%)	<0.001
Number of drugs	2/16 (12%)	14/27 (52%)	0.009

CR for cytopenia: Hb 80–100 g/L (NCI-CTCAE grade 2 anemia) and/or platelet count >100×10^9/L and/or neutrophil count >1.5×10^9/L.
PR: red cell transfusion independence and/or platelet count 30–100×10^9/L and/or neutrophil count >1.5×10^9/L.
NR for cytopenia: failure to achieve partial/complete response objectives.
CR for lymphoproliferation: lymphoid organs return to normal size.
PR for lymphoproliferation: lymphoid organs reduce their volume.
NR for lymphoproliferation: failure to achieve partial/complete response objectives.
CR for fever and other symptoms: disappearance.
PR for fever and other symptoms: attenuation.
NR for fever and other symptoms: failure to achieve partial/complete response objectives.
Degree of cytopenia was defined according to NCI-CTCAE v.4. Available from: https://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf.
[a]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 CR, PR, or NR.
AIHA = autoimmune hemolytic anemia; AIN = autoimmune neutropenia; ALPS = autoimmune lymphoproliferative syndrome; CR = complete response; DNTs = double-negative T cells; ITP = immune thrombocytopenia; MMF = mychopenolate-mophetil; NR = nonresponse; PR = partial response.

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 rapamycin (the second-line treatment) versus 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 upfront therapies in documented classical ALPS.[31]

Also biological phenotype was different from ALPS-FAS/CASP10 since ALPS-U patients displayed a significantly lower association with elevated Vitamin B 12 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.[30] Indeed, in our cohort, this panel was significantly associated to ALPS-FAS/CASP10 when all the 4 parameters were

Table 7

Flow Cytometry as a Diagnostic Tools for ALPS Screening

	ALPS-FAS CASP +	ALPS-U	*P*
A) CD3CD4-CD8-+T-CRαβ+ (DN) (>1.5%)	18/18 (100%)	28/28 (100%)	
B) CD19+CD27+ (<15%)	8/11 (73%)	19/26 (73%)	0.98
C) TCR αβ+ B220+ (>60%)	9/11 (82%)	14/26 (50%)	0.10
D) 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

ALPS = autoimmune lymphoproliferative syndrome.

contemporarily positive. Although this may somehow orient clinicians toward ALPS-FAS/CAPS10 versus 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 the ALPS-FAS/CAPS10 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 2 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. The 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 3). The pathogenic role of the detected mutations was known only for a minority of them (4/14). In one additional patient (ALPS-U patient 23), we found an ADA2 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 its pathogenic role for this variant. In all ALPS CASP10 subjects carrying benign/likely benign variants of CASP10, an altered apoptosis induced by FAS-ligand and TRAIL stimulation was shown. In addition to this, we also proved a reduced cleavage of CASP8 and PARP likely reflecting a reduced upstreamed CASP10 activity; thus, indirectly supporting a potential pathogenic role for this variants.[22] The variants of CASP10 have always been reported in the literature with conflicting interpretation; nevertheless, some of them have been recently reported to impaired apoptosis and suggest that could play a role in predisposition of immune dysregulation.[22]

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

VUS have a probable effect on the phenotype. Although taking VUS variants into consideration for diagnostic purpose may be questionable, clinicians, who found VUS in patients showing symptoms and laboratory alterations similar to ALPS-FAS/CASP10 patients, may have an impact.[32]

Still with the bias of having adopted a NGS panel largely composed of immune genes virtually, all of detected variants are related to immunodeficiencies/immune dysregulation diseases. Interestingly, the same was shown in a recent study on a large cohort of pediatric Evans syndrome[33] patients where an association was found with high frequency of potentially damaging variants in immune genes.

The availability of the new genetic techniques such as NGS and Whole Genome Sequencing 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.[33,34] 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 differs from ALPS-FAS/CASP10 because it has a clearer nonhematological autoimmune signature, it is more associated to lymphopenia, to lower response to treatment in general and to drugs classically effective in ALPS-FAS/CASP10 such a MMF and rapamycin, and 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 form ALPS (eg, 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 finings in the context of the clinical phenotype.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. EP designed the research, contributed essential data, cared for the patients and wrote the manuscript. AG, IC, ML performed the genetic analysis. ML performed the IL-18, IL-10, and FAS-ligand tests. PT performed the study of lymphocyte immune phenotype. FT performed functional tests. NC performed the FAS-mediated aptosis test. EM, MC, FP, CM, RM, DG, EF, AM, EM, PC, GR, MP, PF, SC, AB, and UR contributed essential data, cared for the patients. JB discussed the results and critically reviewed the manuscript. MM, CD, and FF designed the study protocol, contributed essential data, cared for the patients, revised the manuscript.

DISCLOSURES

The authors have no conflicts of interest to disclose.

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