

Letter to the Editor

Using biomarkers to predict the presence of *FAS* mutations in patients with features of the autoimmune lymphoproliferative syndrome

To the Editor:

The autoimmune lymphoproliferative syndrome (ALPS) is characterized by chronic lymphadenopathy, splenomegaly, autoimmune cytopenias, and expansion of T cell receptor (TCR) $\alpha\beta^+ CD3^+ CD4^- CD8^-$ ($\alpha\beta$ -double-negative [DNT]) cells (see this article's Table E1 in the Online Repository at www.jacionline.org). Approximately two thirds of the patients with ALPS symptoms are genetically characterized, and most have germline (ALPS Ia) or somatic (ALPS Ia-s) *TNFRSF6* (*FAS*) mutations. A small number of patients have defects in genes encoding Fas ligand (ALPS Ib), caspase-10 (ALPS II), or Neuroblastoma-RAS (NRAS) viral oncogene homolog (ALPS IV). In addition, a large group of patients with ALPS findings remain genetically uncharacterized (ALPS III), and yet another has an undefined ALPS-like syndrome (ALPS-phenotype; Table I).^{1,2} Given the clinical similarities among all these groups, we sought to develop a biomarkers-based algorithm to predict the presence or absence of *FAS* mutations in this setting.

To this end, we investigated 26 parameters including immunophenotyping, eosinophil and monocyte counts, serum or plasma vitamin B12 (B12), soluble *FAS* ligand (sFASL), immunoglobulins, and levels of 14 cytokines in 562 subjects classified into 6 categories (Tables I and E1). The number of measurements, medians, and first° and third° quartiles are presented in this article's Tables E2 and E3 in the Online Repository at www.jacionline.org. A full description of the Methods can be found in the Online Repository at www.jacionline.org.

Elevated $\alpha\beta$ -DNT cells are a hallmark of ALPS, but their utility for predicting *FAS* mutations had not been previously evaluated.³ Patients with ALPS Ia and Ia-s had a high percentage of $\alpha\beta$ -DNT cells, with median values 5.1% and 7.7%, respectively, compared with 0.5% for control mutation-negative relatives (MNRs; $P < .0001$; Fig 1, A; Table E2). The $\alpha\beta$ -DNT level was predictive of *FAS* mutations, with values $>4\%$ found in 60% (90/152) of patients with type Ia and in the majority of patients with type Ia-s (7/9), but in only 13% (11/85) of patients with ALPS type III and ALPS-phenotype (Fig 1, A). This value was associated with a positive likelihood ratio (LR) of 5.0 and a posttest probability of 89.3% for harboring *FAS* mutations. Conversely, the presence of $\alpha\beta$ -DNT cells in the 1% to 2% range decreased the posttest probability to 25%, with a LR of 0.19 (Fig 2, B and C; see this article's Table E4 in the Online Repository at www.jacionline.org).

In line with previous reports, patients with ALPS, regardless of mutation status, had $<16\%$ of circulating B cells expressing the memory marker CD27 (Fig 1, B).⁴ Finding memory B cells $>16\%$ made the diagnosis of ALPS very unlikely (LR = 0.17). Other described abnormalities including increased $CD3^+ HLA-DR^+$ to $CD3^+ CD25^+$ ratio and high number of B cells had no additional diagnostic utility.⁴

We also evaluated serum B12 levels in patients with ALPS and found very elevated median levels in ALPS Ia and Ia-s (2259 ng/L; 1653 ng/L) compared with control MNRs (474 ng/L; $P < .0001$) and healthy mutation-positive relatives (570 ng/L;

TABLE I. Description of patients with ALPS and control groups included in the study

Classification	Description	No. of subjects
ALPS Ia	Patients with ALPS and germline <i>FAS</i> mutations	162
ALPS Ia-s	Patients with ALPS and somatic <i>FAS</i> mutations	9
Healthy mutation-positive relatives	Healthy relatives of patients with ALPS Ia who carry a <i>FAS</i> mutation	115
Mutation negative relatives	Healthy relatives of patients with ALPS Ia who do not carry a <i>FAS</i> mutation.	179
ALPS III	Patients who fulfill criteria for ALPS but have no causative mutation identified	52
ALPS-phenotype	Patients with autoimmune cytopenias, lymphadenopathy/splenomegaly, and $\alpha\beta$ -DNT cells $>1\%$, without genetic abnormalities or lymphocyte defective apoptosis	40

$P < .0001$). A modest but statistically significant increase was also noted in ALPS III and ALPS-phenotype, with medians of 759 ng/L and 943 ng/L, respectively (Fig 1, C). Levels above 1500 ng/L were observed in only 15% (4/28) of the patients with ALPS type III and ALPS-phenotype, contrasting with 63% (72/114) of patients with ALPS Ia. The LR for a *FAS* mutation with B12 levels >1500 ng/L was 4.0, with a posttest probability of 87%. In contrast, having B12 levels <1000 ng/L diminished the posttest probability to 35% (Fig 2, B and C; Table E4).

Analysis of plasma cytokines revealed 2 additional biomarkers for ALPS: IL-18 and TNF- α . Median plasma IL-18 levels were elevated in patients with ALPS Ia and Ia-s compared with control MNRs (1041 pg/mL, 1526 pg/mL, and 208 pg/mL, respectively; $P < .0001$). Patients with ALPS III and ALPS-phenotype had median values of 521 pg/mL and 702 pg/mL, respectively ($P < .001$ compared with MNRs; Fig 1, D). Furthermore, IL-18 <500 pg/mL was rarely seen in patients with ALPS and *FAS* mutations (7/56), with an associated negative LR of 0.19. TNF- α levels were higher in all ALPS groups with median values of 5 pg/mL for ALPS Ia ($P < .0001$), 9 pg/mL for ALPS Ia-s ($P < .05$), 8 pg/mL for ALPS III ($P < .0001$), and 7 pg/mL for ALPS-phenotype ($P < .0001$) compared with 1.3 pg/mL for MNRs (Fig 1, E).

As previously reported, IL-10 was markedly elevated in ALPS Ia and Ia-s compared with MNRs ($P < .0001$) and patients with ALPS III and ALPS-phenotype ($P < .0001$)^{5,6} (Fig 1, F). Sixty percent (83/139) of the patients with ALPS Ia and all patients with ALPS Ia-s exhibited values >40 pg/mL, contrasting with 26% (10/38) of patients with ALPS III and ALPS-phenotype. For levels of IL-10 >40 ng/mL, the positive LR was 3.8, with a posttest probability of 85% for having a *FAS* mutation. Notably, only 20% (29/141) of patients with ALPS Ia and no patients with ALPS Ia-s had IL-10 values <20 pg/mL, giving a negative LR of 0.31 and a posttest probability of 33% for *FAS* mutations (Fig 2, B and C; Table E4).

A recent report documented high levels of sFASL in patients with ALPS.⁷ We expanded these findings analyzing more than 200 patients and controls. Ninety-seven percent of patients with ALPS

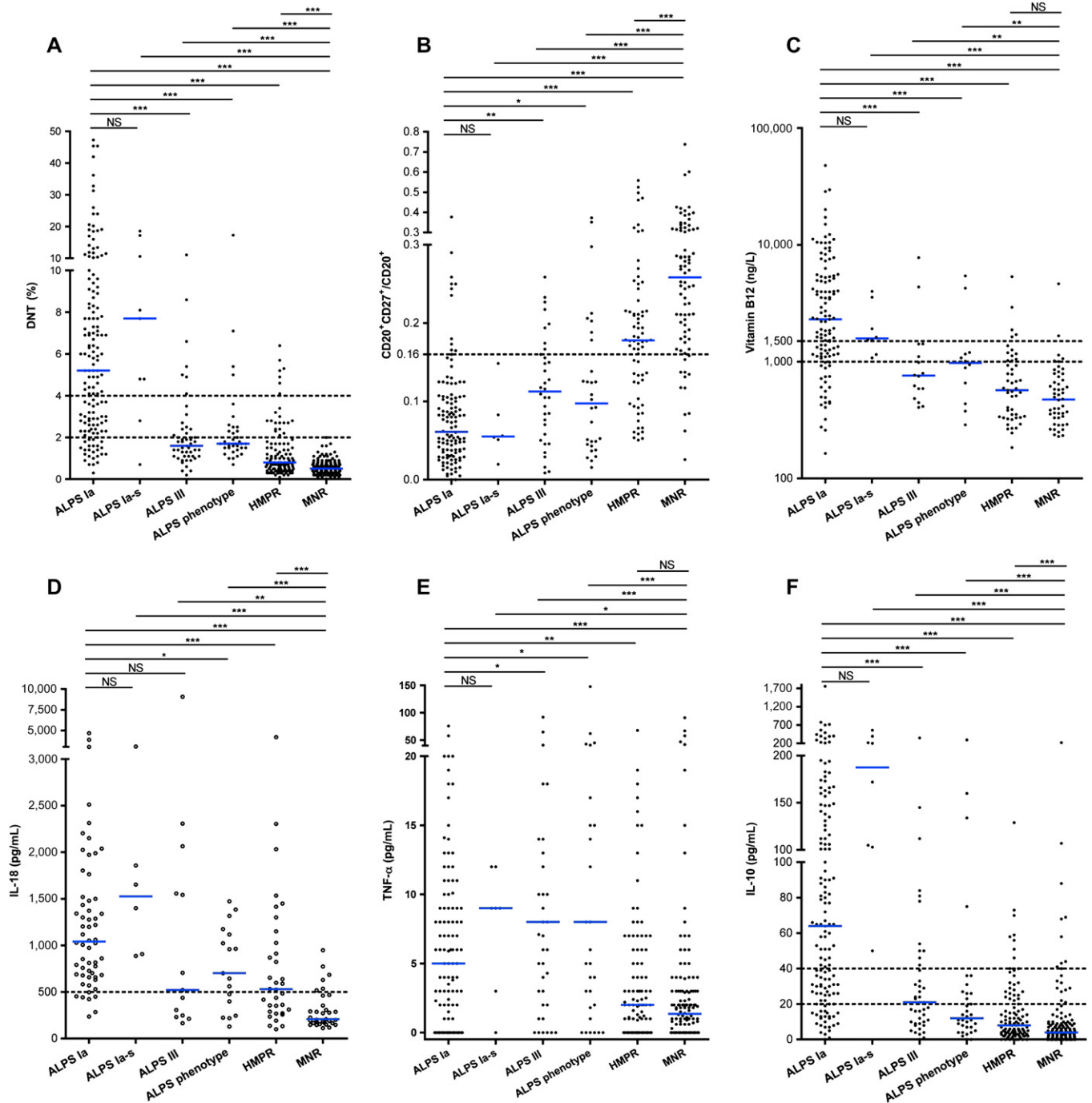


FIG 1. Biomarkers in patients with ALPS and control groups. Dashed lines represent cut-off values used to calculate likelihood ratios. Bars denote median values. P values for the differences between groups were obtained by Mann-Whitney test and are shown above each graph (*** $P < .0001$; ** $P < .001$; * $P < .05$; NS, no significant difference). HMPR, Healthy mutation-positive relative; MNR, Mutation negative relative.

Ia (136/140) and all patients with ALPS Ia-s had plasma sFASL >200 pg/mL, with median values of 1114 pg/mL and 1329 pg/mL, respectively, compared with control MNR levels of 104 pg/mL ($P < .0001$ for both groups). Only modest elevations of sFASL were seen in patients with ALPS III and ALPS-phenotype, as well as healthy mutation-positive relatives, with median values of 208 pg/mL, 174 pg/mL, and 207 pg/mL, respectively (Fig 2, A). These findings make sFASL the most sensitive biomarker to rule out a

FAS mutation, with values <200 pg/mL associated with a negative LR of 0.05 and a posttest probability of 7.7% (Fig 2, C; Table E4). Soluble FASL also showed a strong positive correlation with IL-10 ($r = 0.8$; $P < .0001$) and a moderate correlation with $\alpha\beta$ -DNT cells ($r = 0.6$; $P < .0001$) and B12 levels ($r = 0.69$; $P < .0001$; see this article's Fig E1, A, in the Online Repository at www.jacionline.org). The area under the ROC curve for sFASL, $\alpha\beta$ -DNT cells, B12, and IL-10 levels were calculated to evaluate how well they

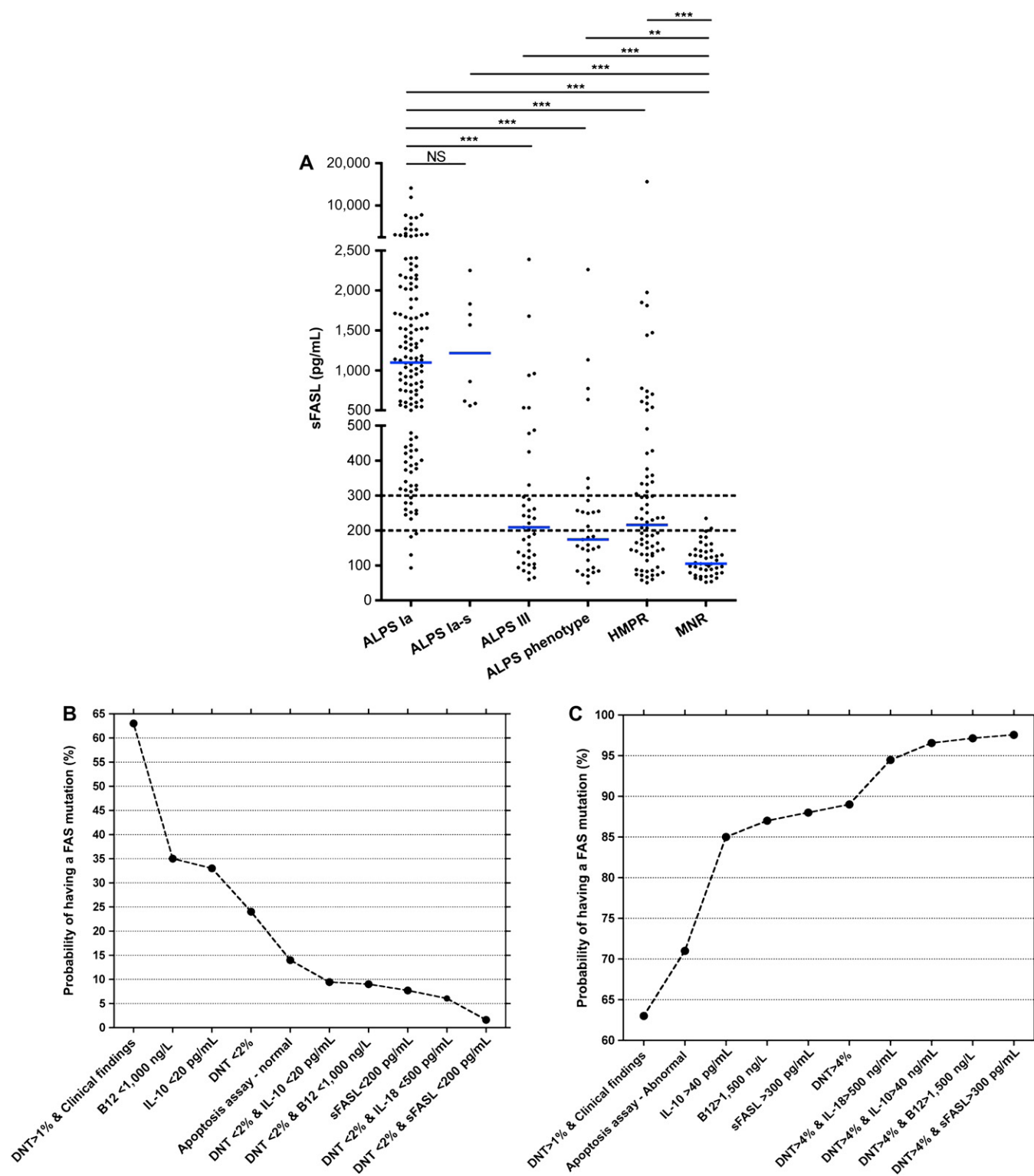


FIG 2. sFASL levels and combinations of biomarkers accurately predict *FAS* mutations. **A**, Scatter plot showing sFASL levels. Increasing **(B)** and decreasing **(C)** probabilities for having a *FAS* mutation according to the percentage of $\alpha\beta$ -DNT cells, levels of vitamin B12, IL-10, IL-18, sFASL, and results of *FAS*-induced apoptosis assay. Bars denote median values. P values for the differences between groups were obtained by Mann-Whitney test and are shown above each graph (*** $P < .0001$; ** $P < .001$; * $P < .05$; NS, no significant difference). *HMPR*, Healthy mutation-positive relative.

discriminate patients with a *FAS* mutation from those without (Fig E1, B). The area under the curve for sFASL was 0.9 (defines an excellent test) and for $\alpha\beta$ -DNT cells was 0.81. B12 and IL-10 exhibited areas significantly less than sFASL ($P < .05$), with values of 0.76 and 0.77.

We next evaluated whether combinations of $\alpha\beta$ -DNT cells, B12, IL-10, IL-18, and sFASL would have increased power to predict or exclude *FAS* mutations in patients suspected of ALPS (Fig 2, B; Table E4). The combination of $\alpha\beta$ -DNT cells $>4\%$ with B12 >1500 ng/L or IL-10 >40 pg/mL or IL-18 >500 ng/mL or sFASL >300 pg/mL was associated with $>95\%$ probability of having a *FAS* mutation. Conversely, having $\alpha\beta$ -DNT cells $<2\%$ in combination with IL-10 <20 pg/mL or B12 <1000 ng/L or IL-18 <500 ng/mL decreased the probability of a *FAS* mutation to less than 10% (Fig 2, C; Table E4). Finally, finding $\alpha\beta$ -DNT cells $<2\%$ and sFASL <200 pg/mL resulted in $<2\%$ probability for a *FAS* mutation.

In conclusion, the biomarkers described should aid in the selection of patients with findings of ALPS for further diagnostic workup. In addition, the presence of a combination of markers strongly suggestive of a *FAS* mutation in the setting of a negative genetic test should prompt a search for somatic mutations in sorted $\alpha\beta$ -DNT cells.

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METHODS

Subjects

Five hundred sixty-two subjects studied at the National Institutes of Health under an Institutional Review Board–approved protocol were included in this study. All patients presented with lymphadenopathy and/or splenomegaly and $\alpha\beta$ -DNT cells above 1% of the total lymphocytes, typically associated with autoimmune cytopenias.

Biomarkers

Immunophenotyping, mutation analysis, and apoptosis assays were performed as previously described.^{E1-E3} Vitamin B12 levels, immunoglobulin measurements, and monocyte and eosinophil counts were obtained by reviewing medical and laboratory records of all participants. For patients with repeated measurements, the first available result was used, regardless of the clinical status or ongoing therapy.

The quantification of plasma ILs was performed by ELISA following the manufacturer's instructions. sFASL, TNF- α , IL-2, IL-7, IL-13, and IL-15 kits were from R&D Systems (Minneapolis, Minn); IL-18 was from MBL Co, Ltd (Naku-ku Nagoya, Japan); IL-10, IL-1b, IL-4, IL-5, IL-6, and IFN- γ kits were from Endogen; IL-12 was from Biosource International (Camarillo, Calif); and IL-23 from Bender Medsystems (Vienna, Austria). The measurements were performed on frozen plasma samples, and for patients with more than 1 sample available, the first collected sample was used for testing. One technical aspect worth noting is that IL-18 levels are spuriously increased in blood samples shipped overnight, especially in the warm summer months, so that only fresh samples (<8h post drawing time) should be used for analysis.

Statistical analysis

Statistical analysis was performed by using Prism (Graphpad Software, San Diego, Calif), JMP-7 (SAS, Cary, NC) and the statistical and graphics language R version 2.9.0 (<http://www.R-project.org>). The Wilcoxon-Mann-Whitney test was used to compare groups. Sensitivity and specificity of each biomarker to detect ALPS and *FAS* mutations were obtained, and LRs ($LR^+ = \text{sensitivity}/100 - \text{specificity}$; $LR^- = 100 - \text{sensitivity}/\text{specificity}$), odds ratios (odds posttest = odds pretest $\times$ LR), and probabilities ($[\text{odds}/1 + \text{odds}] \times 100$) were calculated. Pretest odds and probability for having a *FAS* mutation were obtained by calculating the prevalence of *FAS* mutations on our whole cohort of patients and were found to be 1.7 and 63%, respectively. When associating more than 1 biomarker, the posttest odds were calculated by multiplying the pretest odds by the combination of LRs (odds posttest = odds pretest $\times$ $LR_1 \times LR_2 \times \dots \times LR_n$). Receiver operating characteristic curves were used to evaluate the best levels of vitamin B12, IL-10, sFASL, and $\alpha\beta$ -DNT able to differentiate *FAS* mutants from no mutants and to evaluate the accuracy of the biomarkers.

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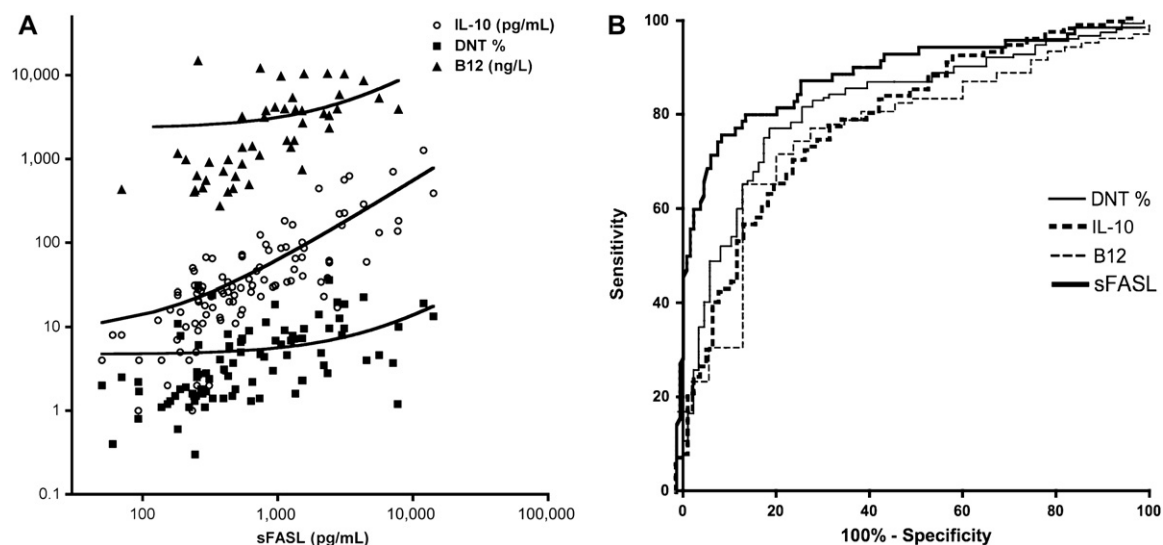


FIG E1. **A**, Correlation plots and curves of sFASL and vitamin B12, IL-10, and $\alpha\beta$ -DNTs. **B**, Receiver operator characteristic curves for $\alpha\beta$ -DNTs, sFASL, IL-10, and vitamin B12.

TABLE E1. National Institutes of Health diagnostic criteria for ALPS

Required criteria
Chronic nonmalignant lymphadenopathy and/or splenomegaly
CD3 ⁺ TCRαβ ⁺ CD4 ⁺ CD8 ⁻ (DNT) cells above 1% of the total lymphocytes
Lymphocyte apoptosis defect
Supporting criteria
Family history of ALPS
Characteristic histopathology
Autoimmune manifestations

TABLE E2. Medians and first and third quartiles of the biomarkers evaluated

Biomarker	N	ALPS Ia		ALPS Ia-s		ALPS III		ALPS-phenotype		HMPR		MNR	
		Median	1st/3rd quartile	Median	1st/3rd quartile	Median	1st/3rd quartile	Median	1st/3rd quartile	Median	1st/3rd quartile	Median	1st/3rd quartile
DNT (no.)	162	101	48/239	9	94	49	30	38	39	106	16	134	10
DNT (%)	162	5.1	2.6/8.4	9	7.7	49	1.6	38	1.7	109	0.8	138	0.5
CD20 ⁺ (%)	149	15	8.5/20.6	8	18.4	45	15	35	17.5	108	10.15	138	10.8
CD20 ⁺ CD27 ⁺ /CD20 ⁺	121	0.06	0.03/0.1	6	0.055	35	0.10	31	0.09	66	0.17	79	0.25
CD3 ⁺ CD25 ⁺ (%)	147	16.2	10.2/23.9	8	11.9	46	12.6	36	13.6	106	24.4	131	25.8
CD3 ⁺ HLA-DR ⁺ (%)	147	20	13.9/27.9	8	15	46	14.3	36	14.7	106	11.6	131	9
B12 (ng/L)	115	2259	1151/5216	8	1653	16	759	13	943	46	570	52	474
sFasL (pg/mL)	142	1114	476/1892	8	1329	39	208	31	174	74	207	44	104
IL-10 (pg/mL)	146	58	25/126	8	187	43	17	35	11	100	7	146	4
IL-1 β (pg/mL)	48	0	0/1	2	0	13	0	7	1	43	1	67	0
IL-2 (pg/mL)	143	0	0/5.5	8	0	43	0	35	1	102	0	141	0
IL-4 (pg/mL)	37	0	0/0	2	0	8	0	4	0	25	0	41	0
IL-5 (pg/mL)	33	0	0/2	1	0	9	0	5	0	39	0	71	0
IL-6 (pg/mL)	140	0	0/2	8	0.5	42	0	34	0.5	102	0	142	0
IL-7 (pg/mL)	24	2	1/4	1	0.8	9	1	5	2	31	2	37	1
IL-12 (pg/mL)	10	0.29	0/0.9	0	ND	3	1	2	7	14	0	34	0
IL-13 (pg/mL)	38	0	0/8.25	1	0	7	0	7	0	14	0	32	0
IL-15 (pg/mL)	73	1	0/2	5	1	27	1	22	1.5	45	2	47	1
IL-18 (pg/mL)	59	1041	684/1495	6	1526	13	521	17	702	36	530	35	208
IL-23 (pg/mL)	32	183	92/439.7	1	89	12	116	14	343	24	151	13	208
TNF- α (pg/mL)	133	5	0/11	8	9	41	8	34	7	95	2	129	1.3
IFN- γ (pg/mL)	142	0	0/4	8	3.5	43	0	35	1	101	0	142	0
IgG (mg/dL)	109	1480	1070/1930	8	1900	15	1040	13	1190	46	1215	54	1010
IgE (IU/mL)	91	64	11/424	7	29	11	19	9	5	33	20	47	32
Eosinophils (K/uL)	123	0.24	0.12/0.43	8	0.15	31	0.15	27	0.14	67	0.13	83	0.17
Monocytes (K/uL)	118	0.6	0.4/1	8	0.43	31	0.55	28	0.62	69	0.44	87	0.45

HMPR, Healthy mutation-positive relative; MNR, mutation negative relative; N, number of subjects studied; ND, not determined.

TABLE E3. Biomarkers in patients with ALPS types Ib, II, and IV

Biomarker	ALPS Ib	ALPS II	ALPS IV
No. of patients	1	1	1
DNT (no.)	66	838	88
DNT (%)	1.5	27	2.2
CD20 ⁺ (%)	17	14.1	49
CD20 ⁺ CD27 ⁺ /CD20 ⁺	ND	0.2	0.03
CD3 ⁺ CD25 ⁺ (%)	6.6	5.8	18.7
CD3 ⁺ HLA-DR ⁺ (%)	36.7	58.9	4.9
B12 (ng/L)	1457	4086	392
sFASL (pg/mL)	86	165	104
IL-10 (pg/mL)	27	353	9
IL-1b (pg/mL)	1	5	0
IL-2 (pg/mL)	11	0	15
IL-4 (pg/mL)	10	0	ND
IL-5 (pg/mL)	ND	ND	0
IL-6 (pg/mL)	0	ND	6
IL-7 (pg/mL)	ND	ND	4
IL-12 (pg/mL)	ND	ND	ND
IL-13 (pg/mL)	350	ND	ND
IL-15 (pg/mL)	ND	ND	ND
IL-18 (pg/mL)	ND	ND	2678
IL-23 (pg/mL)	ND	ND	ND
TNF- α (pg/mL)	15	6.5	6
IFN- γ (pg/mL)	5	53.6	0
IgG (mg/dL)	417	2710	1750
IgE (IU/mL)	10	ND	5
Eosinophils (K/uL)	0.7	0.66	0.08
Monocytes (K/uL)	1.4	1.059	2.1

ND, Not determined.

TABLE E4. Probabilities, likelihood ratios, and odds ratios for having a *FAS* mutation associated with different biomarker levels

Biomarker(s)	FAS mutation probability (%)	Likelihood ratio	Odds ratio
DNT >1% and clinical findings	63		1.7
Abnormal apoptosis assay	71	1.46	2.5
IL-10 >40 pg/mL	85	3.28	5.6
IL-10 <20 pg/mL	33	0.31	0.49
B12 >1500 ng/L	87	3.99	6.8
B12 <1000 ng/L	35	0.31	0.54
DNT >4%	89	5.00	8.5
DNT <2%	24	0.19	0.32
IL-18 >500 ng/L	77	2.05	3.48
IL-18 <500 ng/L	25	0.19	0.33
sFASL >300 pg/mL	88	4.5	7.7
sFASL <200 pg/mL	7.7	0.05	0.08
DNT >2% and IL-10 >20 pg/mL	88.4	4.5	7.62
DNT >2% and IL-10 >40 pg/mL	93.2	8.06	13.7
DNT >4% and IL-10 >20 pg/mL	93.9	9.16	15.58
DNT >4% and IL-10 >40 pg/mL	97	16.47	27.9
DNT >2% and B12 >1000 ng/L	89.4	4.9	8.40
DNT >2% and B12 >1500 ng/L	94.3	9.8	16.65
DNT >4% and B12 >1000 ng/L	94.5	10.1	17.19
DNT >4% and B12 >1500 ng/L	97	20	33.9
DNT >2% and IL-18 >500 pg/mL	89.52	5.02	8.54
DNT >4% and IL-18 >500 pg/mL	94	10.28	17.47
DNT >2% and sFasL >200 pg/mL	89.3	4.91	8.36
DNT >2% and sFasL >300 pg/mL	95	11.09	18.9
DNT >4% and sFASL >200 pg/mL	94.7	10.05	17.09
DNT >4% and sFASL >300 pg/mL	97	22.7	38.59
DNT <2% and IL-10 <20 pg/mL	9	0.06	0.10
DNT <2% and B12 <1000 ng/L	9	0.06	0.10
DNT <2% and IL-18 <500 ng/L	6.0	0.037	0.06
DNT <2% and sFASL <200 pg/mL	1.6	0.009	0.016