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Key diagnostic markers for Autoimmune Lymphoproliferative Syndrome with molecular genetic diagnosis

Tracking no: BLD-2020-005486R2

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Abstract:
Autoimmune lymphoproliferative syndrome (ALPS) is a rare immunodeficiency caused by mutations in genes affecting the extrinsic apoptotic pathway (*FAS, FASL, CASP10)*. This study evaluated the clinical manifestations, laboratory findings and molecular genetic results of 215 patients referred as possible ALPS. Double negative T-cell (DNT) percentage and *in vitro* apoptosis functional tests were evaluated by FACS; interleukin 10 and 18 (IL-10, -18) and soluble FAS ligand (sFASL) were measured by ELISA. Genetic analysis was performed by next generation sequencing. Clinical background data were collected from patients' records. Patients were categorised into definite, suspected and unlikely ALPS, and laboratory parameters were compared among these groups. From 215 patients, 38 met the criteria for definite ALPS and 17 for suspected ALPS. The definite and suspected ALPS patient population showed higher DNT than unlikely ALPS and had higher rates of lymphoproliferation. Definite ALPS patients had a significantly more abnormal *in vitro* apoptosis function with lower annexin than patients with suspected ALPS ($P=0.002$) and patients not meeting the ALPS criteria ($P<0.001$). The combination of elevated DNT and an abnormal *in vitro* apoptosis functional test was the most useful to identify all types of ALPS patients; the combination of abnormal *in vitro* apoptosis functional test and elevated sFASL was a predictive marker for ALPS-FAS group identification. Lymphoproliferation, apoptosis functional test and DNT are the most sensitive markers; elevated IL-10 and IL-18 are additional indicators for ALPS. The combination of elevated sFASL and an abnormal apoptosis function was the most valuable prognosticator for patients with *FAS* mutations.

Conflict of interest: No COI declared

COI notes: No, none of the authors has a relevant conflict of interest.

Preprint server: No;

Author contributions and disclosures: EM and NR analysed the data and wrote the paper. EM, NR, AZ collected clinical and laboratory results. FE, MH, DS, KG helped to establish the assays and translate into routine service. GK, HA performed the statistical analysis and assisted with the evaluation of molecular analysis AT, FH, MB, SB and KG supervised the work and gained the funding under which this work was performed All authors reviewed and edited the paper

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: Emails to corresponding author

Clinical trial registration information (if any):

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Key diagnostic markers for Autoimmune Lymphoproliferative Syndrome with molecular genetic diagnosis

(short title for the running head): Diagnostic markers in ALPS

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Abstract word count: 250

Text word count: 4938

Table count: 3

Figure count: 3 + 1 supplementary figure

Reference count: 53

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Key points

- In patients with lymphadenopathy and/or splenomegaly with elevated DNT, ALPS must be suspected. Genetics and biomarkers can confirm this.

Abstract

Autoimmune lymphoproliferative syndrome (ALPS) is a rare immunodeficiency caused by mutations in genes affecting the extrinsic apoptotic pathway (*FAS, FASL, CASP10)*. This study evaluated the clinical manifestations, laboratory findings and molecular genetic results of 215 patients referred as possible ALPS. Double negative T-cell (DNT) percentage and *in vitro* apoptosis functional tests were evaluated by FACS; interleukin 10 and 18 (IL-10, -18) and soluble FAS ligand (sFASL) were measured by ELISA. Genetic analysis was performed by next generation sequencing. Clinical background data were collected from patients' records. Patients were categorised into definite, suspected and unlikely ALPS, and laboratory parameters were compared among these groups. From 215 patients, 38 met the criteria for definite ALPS and 17 for suspected ALPS. The definite and suspected ALPS patient population showed higher DNT than unlikely ALPS and had higher rates of lymphoproliferation. Definite ALPS patients had a significantly more abnormal *in vitro* apoptosis function with lower annexin than patients with suspected ALPS (P=0.002) and patients not meeting the ALPS criteria (P<0.001). The combination of elevated DNT and an abnormal *in vitro* apoptosis functional test was the most useful to identify all types of ALPS patients; the combination of abnormal *in vitro* apoptosis functional test and elevated sFASL was a predictive marker for ALPS-FAS group identification. Lymphoproliferation, apoptosis functional test and DNT are the most sensitive markers; elevated IL-10 and IL-18 are additional indicators for

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ALPS. The combination of elevated sFASL and an abnormal apoptosis function was the most valuable prognosticator for patients with *FAS* mutations.

Keywords: Autoimmune Lymphoproliferative Syndrome, FAS, double negative T cell, in vitro apoptosis functional test, lymphadenopathy

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Introduction

Autoimmune lymphoproliferative syndrome (ALPS or Canale-Smith syndrome) is a rare immunodeficiency with several pathognomic hallmarks, such as: non-malignant, chronic lymphoproliferation, unexplained lymphadenopathy, splenomegaly; immune-mediated cytopenia; hypergammaglobulinemia (IgG) caused by an abnormal extrinsic FAS-mediated apoptotic pathway.[1-3] Defective lymphocyte apoptosis could principally cause autoimmune manifestations, mainly multilineage cytopenias and less frequently causing nephritis, hepatitis, uveitis, arthritis or colitis. Furthermore, patients with ALPS and also genetically affected siblings without an ALPS phenotype are predisposed to several malignancies such as solid tumours (e.g. thyroid, breast, and liver) or leukaemia, the risk of non-Hodgkin lymphoma is up to 50 times more frequent compared to the general population.[4-6]

In ALPS, the defective extrinsic apoptotic signalling is caused by germline and somatic mutations in *FAS* (*TNFRSF6, CD95, AP01*), *FASLG* and *CASP10* genes, encoding Fas cell surface death receptor protein, FAS ligand protein and caspase 10.[7] The majority of ALPS patients present with germline heterozygous or somatically acquired *FAS* mutations. Rarely *FAS* mutations are inherited in an autosomal recessive manner.[2,3,8,9] *FAS* gene encodes a protein receptor expressed on B and T lymphocyte lineages. *FASLG* gene mutations are identified in extremely rare cases of ALPS.[10] On the basis of the underlying pathogenic genetic variants, ALPS is classified into four categories, according to the affected genes such as ALPS-FAS, ALPS-FASLG and ALPS-CASP10.[2,7] In ALPS patients without defined mutation in the above-mentioned genes (20-30 % of the cases), the condition is classified as ALPS-U (unknown).[2,6,8,11-17] Patients with pathogenic caspase-8 (*CASP8*) gene

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variants resulting in Caspase-8 deficiency (CED) are classified as an ALPS-related disorder characterised by defective T-, B-, and NK-cell activation in addition to defective FAS-induced lymphocyte apoptosis.[2,10,18]

Impaired activation induced cell death (AICD) in patients suffering from ALPS results in the development of an abnormal T cell repertoire.[19] The accumulation of an autoreactive $CD3^{+}TCR\alpha\beta^{+}$ $CD4^{-}CD8^{-}$ double negative T-cell (DNT) population was initially reported in 1992, as a main characteristic element of ALPS. This cell population normally accounts for about 1% of all T-cell subsets in the paracortical region and peripheral blood in healthy individuals.[20,21] The DNTs resemble normally differentiated T-cells and usually express naïve T cell markers such as CD45RA on their cell surface. However, these cells are highly proliferative and are able to induce up-regulation in mammalian target of rapamycin (mTOR) and several other growth pathways.[15,22] Skewed T- helper cell profile, and reduced number of CD27+ memory B cells have been described. Other consequential laboratory features of ALPS are raised levels of soluble FAS ligand (sFASL), interleukin-10 and -18 (IL-10 and IL-18).

This retrospective study aims to review the ALPS-2010 diagnostic criteria[3,23] and define the most predictive and useful biomarkers, and their combinations by evaluating 215 patients with clinical evidence of ALPS regarding clinical manifestation, laboratory findings and molecular genetic results.

Methods

Cohort description

Between 2008-2018, blood samples from 215 patients (132 males and 83 females, with a median age of 12.3 years, range: 1 month - 76 years) with a clinical suspicion

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of ALPS were referred to the Immunology Laboratory, Great Ormond Street Hospital (GOSH), London, United Kingdom. Patients belonged to 197 unrelated families. Clinical data included information about the presence of chronic non-malignant, non-infectious lymphadenopathy and/or splenomegaly, cytopenia, immunoglobulin G (IgG), vitamin B12 levels. The GOSH Immunology Laboratory assessed the DNT cells, performed a lymphocyte apoptosis function assay and measured sFASL, IL-10 and IL-18. Genetic analysis was performed in North East Thames Regional Genetics Laboratory, London, United Kingdom. Clinical, immunological and genetic laboratory data were collected retrospectively and available clinical outcome data are also included. This study was approved by Bloomsberry Research Ethics Committee and was conducted in accordance with the Declaration of Helsinki.

Assessment of laboratory parameters

The measurement of $CD3^{+}TCR\alpha\beta^{+}$ $CD4^{-}CD8^{-}$ double negative T-cells (DNT) were performed by flow cytometry. The pathogenic level of DNT has been defined as greater than 1.8% of all alpha/beta T-cells based on the gating strategy of the laboratory.[2,24]

The *in-vitro* FAS-mediated apoptosis assay utilized peripheral blood mononuclear cells (PBMC) stimulated for six to seven days with anti-CD3 and IL-2. After incubation, cells were stimulated with anti-FAS antibody to trigger apoptosis. Apoptosis can be detected by the surface staining of cell membrane proteins (Annexin V). Apoptotic cells positive for Annexin V but negative for the DNA viability stain (7AAD) were enumerated by flow cytometry. In normal individuals the difference between the anti-FAS antibody stimulated sample and unstimulated sample was greater than 3.0 fold change.[24] Annexin V expression post anti-FAS antibody

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treatment was usually at a low level in ALPS patients. The assessment of healthy unrelated control samples were undertaken with each patient sample as quality control for the variables that may affect this functional assay (e.g. transport, temperature, reagents). The normal Annexin V fold change was defined as greater than 3.0, defective apoptosis was defined as lower than 2.0-fold change and the range between 2.0-3.0 was described as equivocal.[2,3]

Human IL-10 Quantikine ELISA Kit (D1000B), Human IL-18 Quantikine ELISA Kit (DL180) and Human FAS Ligand/TNFSF6 Quantikine ELISA Kit (DFL00B) were used from the R&D Systems. The normal ranges were defined lower than 40 pg/ml, 500 pg/ml, 200 pg/ml respectively. The normal range of vitamin B12 was between 228-1510 pg/mL and the normal range of IgG was within 5.4-16.1 g/L.

Genetic studies

Genes affecting the extrinsic apoptotic pathway (*FAS, FASLG, CASP10*) and a further 79 genes included in the targeted immunodeficiency and gastrointestinal enrichment (TIGER) panel were analysed by next generation sequencing (NGS).[25] The list of sequenced genes is available in Supplementary Table 1. The library preparation was done using Sure Select XT custom kit followed by sequencing on Illumina MiSeq. Detected variants were evaluated based on the recommendations of the American College of Medical Genetics and Genomics (ACMG).[26] Web-based VarSome (www.varsome.com) helped variant annotation and classification.[27] Potential splice variants were evaluated by Human Splicing Finder software (http://www.umd.be/HSF/).[28] Identified variants were confirmed by Sanger sequencing.

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Applied diagnostic evaluation

The clinical symptoms and laboratory biomarkers were evaluated by the ALPS-2010 diagnostic protocol,[3,23] which defines four major criteria, including: chronic non-malignant lymphoproliferation (>6 months) with splenomegaly and/or lymphadenopathy; DNT elevation (1.8%) in peripheral blood; defective in vitro FAS-mediated apoptosis; and recognisable genetic pathogenic mutation (in *FAS, FASLG, CASP10* genes). Along with these major criteria, there are six minor criteria, including multilineage cytopenia, elevated levels of IgG, IL-10, IL-18 or vitamin B12 level in serum and increased sFASL level in plasma. At least three major criteria or two major and two minor criteria are required for a diagnosis of definite ALPS. The diagnostic protocol was updated according to the working definitions for clinical diagnosis of primary immunodeficiencies of the European Society for Immunodeficiencies (ESID)[29], where elevated DNT ratio was only a major, but not a required criterion. The diagnostic protocol was extended: suspected ALPS was defined as the fulfilment of two major and one minor criteria. This group was created to include those patients who have possibly received immunosuppressive treatments at the time of the referral. Cases lacking information about at least two major diagnostic and at least one minor criterion were defined as non-evaluable.

Statistical analysis

For the analysis of continuous variables, Shapiro–Wilk test was used to test for normality. None of the parameters examined showed normal distribution thus Kruskal–Wallis or Mann-Whitney-U tests were performed with Dunn's test for post hoc analysis. For the analysis of categorical variables, we applied Pearson's chi-

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squared test. Data were analysed using IBM SPSS Statistics 23, Prism GraphPad and Microsoft Excel. Figures were made using Adobe Illustrator.

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Results

Clinical and laboratory features

Out of the 215 patients referred with clinical evidence of ALPS diagnosis, clinical background was recorded for 87 patients including lymphoproliferation, lymphadenopathy, splenomegaly and for 101 patients' haemoglobin, platelet and white blood cell counts were available. DNT was available for 146 cases, apoptosis functional test was performed for 192, genetic results were accessible for 86 patients. (Figure 1). In addition, immunoglobulin G (IgG) (n=66), vitamin B12 levels (n=31), sFASL (n=126), IL-10 and IL-18 (n=30) were also measured. Patients were categorized as non-evaluable in 75 cases. From the evaluable patient group, 38 patients (27.1%) met the criteria of definite ALPS (median age at first referral: 9.3 years, range: 4 month-77 years; Supplementary Figure 1). Another 17 patients (12.1%) fulfilled the criteria of suspected ALPS (median age at first referral: 13.1 years, range: 1 month-19 years, Supplementary Figure 1), while 85 patients (60.7%) did not meet the ALPS diagnostic criteria (unlikely ALPS group, median age at first referral: 10.3 years, range: 2 month-64 years).

The clinical criteria of ALPS including chronic lymphoproliferation with or without lymphadenopathy/splenomegaly occurred more frequently in definite (97.1%, n=33/34) and suspected (87.5%, n=14/16) ALPS groups than in unlikely ALPS (55%, n=22/40, ****P<0.0001). Multilineage cytopenia was observed in 69% (n=20/29) of definite, in 56.3% (n=9/16) of suspected and in 56.6% (n=30/53) of unlikely ALPS population (P= 0.5168, Figure 2A). Outcome data was available in a limited number of patients. Out of 34 definite ALPS patients 3 (3/34, 8.82%) and another 3

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suspected ALPS patients (3/16; 19.75%) underwent splenectomy. Lymphoma development was described in two (2/34; 5.88%) definite ALPS patients.

Abnormally high DNT were observed in all definite (n=34) and in all suspected ALPS patients (n=14). In the unlikely ALPS population 35 out of 68 (51.5%) cases showed DNT elevation. The median DNT ratio was 3.95% (range: 1.8-23.0%) in definite, 2.6% (1.9-6.7%) in suspected and 1.85% (0.0-13.9%) in the unlikely ALPS groups. There was significant difference between definite ALPS and unlikely ALPS groups (****P<0.0001) and between suspected ALPS and unlikely ALPS groups (*P=0.0496, Figure 2B).

In the definite ALPS group (n=33), 15 patients had an abnormal apoptosis functional test (45.5%), 11 had equivocal (33.3%) and 7 had a normal result. While in the suspected ALPS group (n=16), two (12.5%) patients had abnormal, two (12.5%) had equivocal and 12 patients had normal apoptosis function. In patients with unlikely ALPS diagnosis (n=80), 3 and 8 patients (3.8 and 10%) had abnormal or equivocal apoptosis functional test results. In the definite ALPS group, the median of annexin expression change was 2.1-fold (range: 0.4-6.7). In suspected ALPS, median of 4.8-fold (1.3-18.1), while in the unlikely ALPS group median of 5.2-fold changes (1.1-30.5) were observed (**P=0.0019 between definite and suspect; ****P<0.0001 between definite and unlikely ALPS groups). However, the suspected and the unlikely ALPS groups did not show significant difference (P=0.8953, Figure 2C).

The sFASL levels showed significant difference between definite and unlikely ALPS groups (**P=0.0013; definite ALPS: median 195, range: 44->1000 pg/ml; suspected ALPS: median 154, 23.5-939 pg/ml; unlikely ALPS: median 139, range: 35->1000 pg/ml, Figure 2D). IL-10 level shows tendency of being elevated in definite ALPS (median 41.8, range: 19-169.2 pg/ml) compared to suspected ALPS (median 24.2,

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20.5-27.9 pg/ml) and unlikely ALPS (median 23.8, range: 8-137.6 pg/ml, P=0.0624; Figure 2E). Additionally, a tendency towards IL-18 level increase was also observed between the groups (definite ALPS: median 909, range: 265-3255 pg/ml vs. suspected ALPS: median 642, 157-1127 pg/ml, unlikely ALPS: median 398, range: 206-1375 pg/ml, P=0.0615; Figure 2F).

Description of biomarker combinations

Optimal biomarker combinations were tested by setting up groups of two where both markers were either positive or either of them was negative; the marker combinations were compared between definite and unlikely ALPS groups (Figure 2G). The combination of DNT and abnormal *in vitro* apoptosis functional test was positive in 79.3% (23/29) in definite ALPS patients and it was negative in 93.7% (59/63) of unlikely ALPS patients; therefore, the sensitivity of this combination was 79.3% and the specificity was 93.7%. The DNT and sFASL combination was positive in 41.9% (13/31) of definite ALPS and it was negative in 87.2% (41/47) of unlikely ALPS patients; and thus the sensitivity was 41.9% and the specificity was 87.2%. The *in vitro* apoptosis functional test and sFASL combination was positive in 36.7% (11/30) of definite ALPS, while it was negative in 96.4% (53/55) of unlikely ALPS patients; therefore the sensitivity of this combination was 36.7% and the specificity was 96.4%. All three combinations showed significant difference between the definite and the unlikely ALPS groups. (****P<0.0001 for DNT and in vitro apoptosis functional test; **P=0.0040 for DNT and sFASL; ***P=0.00012 for in vitro apoptosis functional test and sFASL).

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Genetic results

Genetic results were available for 87 patients. We considered pathogenic, likely pathogenic and variants of unknown significance (VUS), classified according to the ACMG guidelines as genetic variants that possibly alter function (Table 1). Variants with higher populational frequencies than expected for ALPS occurrence, or variants that do not alter protein function (intronic not affecting splice site, exonic synonymous or categorized as neutral/tolerated by *in silico* prediction, such as Provean, SIFT, MetaSVM) were referred to as likely benign/benign genetic variants. Previous clinical and *in vitro* functional studies considering gene variants were reviewed.

FAS gene variants were identified in 21 patients, 17 of them were considered as having a potentially functional variant (14 definite and 1 had suspected ALPS). The remaining two cases did not have enough clinical/laboratory data for evaluation (one individual was identified during family screenings of affected proband). Two frameshift and two nonsense pathogenic variants were identified in heterozygous forms in four definite ALPS patients. Three of these loss of function mutations have not been reported before in the literature (c.715_721delGTCATGA, p.Val239HisfsTer2; c.719_722delinsAGTTA; p.Met240LysfsTer7 and c.76C>T, p.Gln26Ter), while one of the nonsense variants was described (c.219C>A; p.Cys73Ter).[4,30] Regarding likely pathogenic missense *FAS* variants three novel (c.742T>G, Phe248Val; c.794A>G, Asp265Gl; c.826C>A, Gln276Lys) and two previously reported (c.749G>A, Arg250Gln; c.776T>G, Ile259Arg) [31,32] were identified. Interestingly, two patients with previously described likely pathogenic missense mutations showed normal *in vitro* FAS-mediated apoptosis assay. The intronic c.335-9_335-6delATTT variant was categorised as VUS (not identified in general population, in ALPS patients, its intronic location does not affect conserved

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splice region). The variant lies in the close proximity to an acceptor splice site, the *in silico* splice prediction suggested that the variant altered splice site, most probably affecting splicing.[28]

The following benign *FAS* gene variants were identified. The *FAS* c.136A>C (p.Thr46Pro) was categorised as a VUS according to ACMG criteria. The variant was found in a 2-year old patient not showing characteristic biomarker positivity of ALPS, which reduced, but did not exclude, the pathogenic role of this genetic variant in ALPS. Further development of ALPS manifestations later in life cannot be excluded. One *FAS* synonymous variant (c.642T>C) and 5 *FAS* intronic variants were found in 3 patients, but minor allele frequencies of these single nucleotide polymorphisms (SNP) are much higher in the normal population, than the expected disease frequency of ALPS (The synonymous variant also co-occurred with a variant previously reported as pathogenic in *CASP10*.) Somatic FAS mutations were not detected in our evaluable patient group with sufficient clinical data. This may reflect a lack of sensitivity of the sequencing assay as genetic analysis was not performed on sorted DNT cells.

In the pathogenic *FAS* variant patient group, 14 patients had abnormal DNT levels and for 3 patients, results were not available. Nine patients had abnormal, 2 had equivocal and 3 had normal in vitro lymphocyte apoptosis functional test. In the other three cases no results were available.

CASP10 variants were found in 5 patients (2 definite, 1 suspected, and 2 non-evaluable). *CASP10* variant (c.1216A>T; p.Ile406Leu) has been previously published as pathogenic in ALPS, in vitro functional studies proved its pathogenicity, although its minor allele frequency (MAF: 0.4%) and in silico prediction programmes defined as benign variant (ACMG categorisation: likely benign) [33]. *CASP10* p.Ile406Leu

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occurred in 2 probands (with definite and suspected ALPS diagnosis) and 2 family members, who were non evaluable. *CASP10* c.295A>G (p.Lys99Glu) was found in one patient with definite ALPS. The variant has not been previously reported in association with ALPS, and has low MAF in general population (0.04%). As the *in-silico* analyses were inconclusive, ACMG classification was likely benign. *CASP10* c.1228G>A (p.Val410Ile) was found in one patient and co-occurred with another missense *CASP10* variant (c.1216A>T) in our cohort, supporting the prediction as a benign variant [29,34,35]. ALPS accompanied by possibly pathogenic or benign *CASP10* variants, had an elevated percentage of DNT (n=2), but a normal *in vitro* lymphocyte apoptosis functional test and sFASL. The clinical manifestations and laboratory markers of patients with potentially pathogenic, novel *FAS* and *CASP10* variants are listed in Table 2. *FASLG* and *CASP8* pathogenic mutations were not identified in our definite or suspected patient cohort.

In our ALPS-U patient group, the TIGER NGS panel for immunodeficiency genes identified pathogenic, likely pathogenic or unknown significant variants in 6 definite and 4 suspected ALPS patients (Table 3). The following genes were affected in our patient cohort: inhibitor of nuclear factor kappa B kinase regulatory subunit gamma (*IKBKG*), ORAI calcium release-activated calcium modulator 1 (*ORAI1*), myosin VB (*MYO5B*), perforin 1 (*PRF1*), recombination activating 1 (*RAG1*), signal transducer and activator of transcription 3 (*STAT3*), TNF receptor superfamily member 13B (*TNFRSF13B*). Lymphoproliferation and elevated DNT were observed in all cases with available data.

Comparison of ALPS-FAS with ALPS-U

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In the combined suspected and definite ALPS groups, ALPS-FAS patients (n=13/17) had significantly higher DNT levels (median 7.5%, range: 4.5-23%) compared to ALPS-U patients (32/35) (median 2.7%, range: 1.8-11%; ****P< 0.0001, Figure 3A). The *in vitro* apoptosis functional test was more impaired in ALPS-FAS (n=14/17; median: 1.6, range: 0.4-3.5) than in ALPS-U (n=31/35; median 3.1, range: 1.3-18.1, ****P< 0.0001, Figure 3B). Even though sFASL proved to be a highly predictive biomarker for ALPS-FAS (n=16/17; median >1000 pg/ml; range: 128.9->1000 pg/ml), in ALPS-U the vast majority of patients showed normal or moderately elevated biomarker level (n=29/35; median 152 pg/ml; range:23.5-486 pg/ml; ****P< 0.0001, Figure 3C).

No significant difference was observed regarding IL-10 (ALPS-FAS: n=4/17; median 96.8 pg/ml, range: 19.9-169.2 pg/ml versus ALPS-U: n=15/35; median: 36.8 pg/ml; range: 19-167.3 pg/ml; P=0.3070), while a tendency toward increased IL-18 was found in ALPS-FAS (n=9/17; median 1380pg/ml, range: 265-3255 pg/ml) compared to ALPS-U (n=13/35; 570 pg/ml; range: 157-3180 pg/ml; P=0.0514, Figure 3D, 3E).

Examining the marker combinations, DNT and *in vitro* apoptosis functional tests showed the highest sensitivity (90.9%) to differentiate between ALPS-FAS and ALPS-U with a negative predictive value of 93.8%. On the other hand, the *in vitro* apoptosis functional test and sFASL combination showed the highest specificity of 92% with the highest positive predictive value of 83.3% for ALPS-FAS. All three combinations showed significantly higher frequency of abnormal results in the ALPS-FAS group (*P=0.01465 for DNT and *in vitro* apoptosis functional test; ***P=0.0002 for DNT and sFASL; ****P<0.0001 for sFASL and in vitro functional apoptosis test, Figure 3F).

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Discussion

ALPS is a clinically and genetically heterogeneous disease with non-specific signs and symptoms. After its description in 1992, several diagnostic criteria were developed to identify the characteristics of this rare disease. All diagnostic protocols from 2000 to date include similar clinical features and biomarkers with different combinations and priorities.[2,3,23,36,37] The latest Working Definitions for Clinical Diagnosis of ALPS published in 2019 by ESID permits a wide range of biomarker combinations, and does not mention strictly required criteria such as DNT elevation or lymphoproliferation.[37] Therefore, this diagnostic protocol allows the potential identification of patients with pre-symptomatic or mild disease, or even of those patients who received previously immunosuppressive treatment. DNT, *in vitro* lymphocyte apoptosis functional test, sFASL, IL-10 could be in the normal or in the moderately abnormal range during or after immunosuppressive medications.[38,39] Hence, we extended the diagnostic protocol with the category of suspected ALPS (two major and one minor criterion were observed) to include all of those patients whose manifestation resembled ALPS; even if their biomarkers were moderately abnormal or unavailable. The limitations of our study are that clinical outcome data were collected and biomarkers were analysed at the time of patient referral, therefore incidence of splenectomy and lymphoma development over time cannot be assessed and the possibility of previous or ongoing immunosuppressive medication cannot be ruled out.

Our findings support the generally accepted diagnostic protocols[2,3,37] that lymphoproliferation (with/without splenomegaly and/or lymphadenopathy) is the most important clinical manifestation of ALPS and presented in 97.1% of our definite ALPS

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population similar to other previous publications.[5] Furthermore, all definite and suspected ALPS patients had elevated DNT, when the test was performed.[2,3,37] However, 51.5% of patients with unlikely ALPS diagnosis also showed abnormal DNT levels and thus, the specificity (50%) and the positive predictive value (PPV) of DNT (43%) proved to be relatively low as previously published.[40] Several types of immunodeficiencies, such as X-linked immunodeficiency with magnesium defect, Epstein-Barr virus infection and neoplasia (XMEN)[41], autoimmune disorders such as juvenile systemic lupus erythematosus, mixed connective tissue disease (MCD) and severe infections[42,43] can also cause elevated DNT.[44] By contrast, the *in vitro* apoptosis functional test showed abnormalities in 78.8% of the definite ALPS, conversely, only 13.8 % of unlikely ALPS population had abnormal apoptosis function. Therefore, this biomarker showed high specificity (92.2%) with high PPV (78.8%). According to our findings the sensitivity of these above-mentioned biomarkers in combination is 79.3% and the specificity is 93.7%. The presence of abnormal levels of these two markers could support the presence of ALPS and the diagnosis of ALPS could be ruled out if both biomarkers are within the normal range. Although the sFASL was significantly elevated in all ALPS populations, its combinations (DNT & sFASL, in vitro apoptosis functional test & sFASL) presented similar specificity, but reduced sensitivity comparing to DNT and *in vitro* apoptosis functional test. IL-18, IL-10, IgG and B12 levels were considered in clinical assessment, but their combinations were not evaluated because of the limited number of cases.

In the suspected ALPS group, beside elevated DNT, the vast majority of biomarkers were moderately abnormal and showed only significant difference in *in vitro* apoptosis function and a tendency in sFASL compared to the definite ALPS

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population (Figure 2C, 2D). These results could be caused by previously commenced immunosuppressive medication or the early development of disease.

In our study investigating a large number of patients with *FAS* gene mutations, 17 cases from 10 independent families were identified. Pathogenic or likely pathogenic *FAS* gene mutations could cause either haploinsufficiency (nonsense and frameshift variants), or disturb the interaction of death receptor FAS and the adaptor protein FADD oligomers. All pathogenic missense mutations including the novel variants in our study affected the death domain of the FAS protein (c.742-c.826) responsible for a strong dominant negative effect.[45] The only non-synonymous missense mutation in our cohort occurring outside of the *FAS* domain (c.136A>G) occurred in an unlikely ALPS patient, reducing the possibility of pathogenicity of the variant (although Varsome category: VUS). Interestingly, early STOP codon producing nonsense *FAS* mutations produced discrepant symptoms in our ALPS patient cohort. For example, patient number 39, had clinical symptoms and laboratory results supporting an ALPS diagnosis except the apoptosis functional assay. However, patient numbers 107 and 49, who also had nonsense *FAS* mutations have impaired apoptosis function. The most probable explanation to this might be that in patient number 39 the mutation affected the extracellular region of the FAS causing a haploinsufficiency, while in, patient numbers 107 and 49 the mutations affect the intracellular region causing a dominant negative effect. It has been previously reported that the intracellular mutations of the FAS receptor have a higher penetrance than extracellular mutations.[4,46] In our study, peripheral blood samples were used for genetic screening, therefore somatic mutations were not analysed in cellular subsets. The coverage of NGS was optimized for germline mutation detection. In the future, it would be desirable to perform *FAS* gene mutation sequencing in sorted double

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negative T cell fractions to differentiate between ALPS with somatic FAS mutation and ALPS-U.

In our cohort 10 out of 37 patients categorized as ALPS-U had variants in genes other than *FAS* or *CASP10*. The vast majority of genes identified in our ALPS-U cohort were previously described in connection with immunodysregulation. Out of the identified genes in our ALPS-U cohort, *IKBKG*, *ORAI1, MYO5B, PRF1, RAG1* gene products do not interact with the Fas-mediated apoptotic pathway according to currently available data. *IKBKG* and *ORAI1* genes play role in the development of ectodermal dysplasia with immunodeficiency. Interestingly, elevated DNT were noted in a patient with *IKBKG* mutation, yet apoptosis functional impairment was not described in such patients previously.[47] *MYO5B* gene is related to microvillus inclusion disease characterised by gastrointestinal and neurological symptoms, but not related with lymphoproliferation. *PRF1* gene mutations are responsible for familial hemophagocytic lymphohistiocytosis (HLH), a syndrome sharing common clinical signs with ALPS such as splenomegaly and cytopenia. Of note, *PRF1* c.272C>T and c.755A>G found in our ALPS-U patients are both described as fairly common variants in the healthy population with minor allele frequency of 2.9% and 0.5% respectively. *RAG1* likely pathogenic variant (c.2290C>T) is associated with Omenn's syndrome, an autosomal recessively inherited form of severe combined immunodeficiency (SCID). Compound heterozygous *RAG1* mutations have also been reported in Evans syndrome.[48] Interestingly our patient was homozygous for the RAG1 variant. The pathogenic gain of function mutation in *STAT3* (c.2144C>T), identified in two unrelated patients in our cohort) leads to lymphoproliferation, autoimmunity, and recurrent infections,[49] the same mutation was independently described in Evans syndrome.[48] *TNFRSF13B* c.310T>C a common functional

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variant, influencing receptor ligand binding and signalling, occurs in heterozygous forms in 2-5% of common variable immunodeficiency (CVID) and in 0,5%-1% in healthy individuals.[50] As described by Teachey et al.[3], CVID patients can present with ALPS-like features.

All patients with ALPS-FAS showed significantly abnormal levels of the majority of recommended laboratory markers, such as DNT, apoptosis functional test, sFASL, similarly to previous studies.[38,40,51] However, patients belonging to the ALPS-U group showed mostly normal apoptosis function and levels of sFASL. This supports the previous finding that ALPS-U patients do not have a detectable abnormal laboratory marker, which would support the presence of pathogenicity in the extrinsic apoptotic pathway. In addition, the elevated level of *in vitro* apoptosis functional test and sFASL could presumptively predict the ALPS-FAS diagnosis, because the specificity is 92% and the PPV is 83.3% (Figure 3F).

Pathogenicity predictions applied by the ACMG criteria were rather contradictory in the case of CASP10 missense variants. The occurrence of the variants in the general population with a considerable minor allele frequency (MAF) of 0.04-4% and the inconsistent, but mostly neutral/tolerated *in silico* functional predictions resulted in likely benign or benign ACMG classification. Supporting this prediction, *CASP10* c.1216A>T (MAF 4% in the Danish population [34]) co-occurred with another missense *CASP10* variant (c.1216A>T) in our cohort. *CASP10* c.1216A>T was considered as a VUS/likely pathogenic variant in our patient cohort as *in vitro* functional studies proved defective apoptosis in ALPS patients.[29] No literature have previously described the *CASP10* c295A>G variant. Biomarker data on ALPS-CASP10 patients are scarcely available, interestingly none of our patients show defective *in vitro* apoptosis functional assaya and elevated sFASL. However, this tendency was not

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confirmed statistically due to the small number of patients. Patients with *CASP10* mutations did not show any abnormalities of *in vitro* apoptosis function. Therefore, the usefulness of the FAS-mediated apoptosis assay and the measurement of sFASL level could be questioned in the ALPS-CASP10 group and would need further investigations. In line with previous observations, the lack of pathogenic *FASLG* and *CASP8* variants in our patient cohort further support the rare occurrence of genetic defects in these genes.[2,10,18]

Our findings illustrate that both major and minor biomarkers defined in the New Diagnostic Protocol 2010 are helpful in ALPS diagnosis. However, not all of them show significant abnormalities in all types of ALPS. Those patients who do not present with lymphadenopathy and/or splenomegaly and do not have increased level of DNT are unlikely to have ALPS. As measurement of DNTs is readily available, these should be assayed as the first investigation in patients suspected of having ALPS. If normal, except in cases of high clinical suspicion, further investigation is not required. Several other non-immunological parameters, such as B12 or high-density lipoprotein have been investigated as useful biomarkers.[40,51] The abnormal synthesis and release of haptocorrin, one of the B12-vitamin transport-proteins, from ALPS lymphocytes are responsible for the high B12 in ALPS, [52] while elevated IL-10 was reported as the direct cause for serum lipoprotein-alterations.[53] As a limitation of our study, these non-specific, but easily available biomarkers (such as high density lipoprotein) were not recorded in all cases, therefore analyses could not be conducted here.

Our findings support that elevated DNT level is an essential major, but not a required criterion for ALPS, if the measurement of DNT is not available. The use of the biomarkers' combinations (DNT, *in vitro* apoptosis functional test and sFASL) could

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accelerate the confirmation or exclusion of an ALPS diagnosis, which is especially useful when molecular analysis in also not available. These reliable blood biomarkers could substitute the molecular analysis by a gene panel that includes *FAS and CASP10* decreasing the cost of diagnosis. The genetic sequencing could be reserved those patients who present uncharacteristic combination of the above-mentioned biomarkers and have high clinical suspicion of ALPS. This diagnostic consideration could facilitate and simplify the rapid diagnosis and treatment of ALPS and decrease the cost of the diagnostic process. The results from this paper should help guide targeted combinations of biomarkers resulting in rapid diagnosis and the optimal use of resources.

Acknowledgments

We wish to acknowledge the staff of the Great Ormond Street Hospital Immunology Laboratory and the North East Thames Regional Genetics Service for helping to analyse these samples. This research has been supported by the Wellcome Trust and by the “National Institute for Health Research Great Ormond Street Hospital UCL Biomedical Research Centre award”. EM and GK were supported by the European Union and the Hungarian Government (EFOP-3.6.3-VEKOP-16-2017-00009).

Authorship Contributions

EM and NR analysed the data and wrote the paper.

EM, NR, AZ collected clinical and laboratory results.

FE, MH, DS, KG helped to establish the assays and translate into routine service.

GK, HA performed the statistical analysis and assisted with the evaluation of molecular analysis

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AT, FH, MB, SB and KG supervised the work and gained the funding under which this work was performed

All authors reviewed and edited the paper

Conflict of Interest Disclosures

The authors declare no relevant conflicts of interest.

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List of Abbreviations:

ACMG - American College of Medical Genetics and Genomics

AICID – Impaired activation induced cell death

ALPS – Autoimmune Lymphoproliferative Syndrome

CED – Caspase-8 deficiency

DNT – Double Negative T-cell

ESID - European Society for Immunodeficiencies

GOSH - Great Ormond Street Hospital

IgG – Immunoglobulin G

MAF – Minor allele frequency

MCD – mixed connective tissue disease

mTOR - mammalian target of rapamycin

NGS - next generation sequencing

PPV - positive predictive value

sFASL – soluble FAS ligand

VUS - variants of unknown significance

XMEN - X-linked immunodeficiency with magnesium defect, Epstein-Barr virus infection and neoplasia

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Figures:

Figure 1: Patients enrolled in the study with the accessible diagnostic results.

215 patients were referred with potential ALPS diagnosis. The minimal criteria for evaluation were at least two major criteria and one minor criterion. 140 patients were evaluable. Patients were considered as definite ALPS patients if at least 3 major or at least 2 major and 2 minor criteria were observed. Patients were considered suspected ALPS if at least 2 major criteria and 1 minor criterion were observed.

Figure 2.: Laboratory markers and clinical parameters of the cohort

(A) Clinical data of the enrolled patients: lymphoproliferation (****P<0.0001 definite vs. unlikely ALPS) and multilineage cytopenia (P= 0.5168). (B) Double negative T-cells (****P<0.0001 definite vs. suspected ALPS, *P=0.0496; suspected vs. unlikely ALPS ALPS). (C) The in vitro apoptosis functional test (****P<0.0001 definite vs. unlikely ALPS group vs **P= 0.0019 definite vs. suspected ALPS). (D) The level of sFASL (**P=0.0013 definite vs unlikely ALPS, P=0.0674 definite vs. suspected ALPS). (E) IL-10 (P=0.0624 definite vs suspected and unlikely ALPS) (F) IL-18 (P=0.0615 definite vs suspected and unlikely ALPS). (G) Assessment of the markers' combination comparing the definite ALPS and unlikely ALPS groups.

All data are presented as median ± interquartile range (IQR).

Figure 3.: Changes of laboratory parameters in ALPS-FAS and ALPS-U

All suspected and definite ALPS patients were categorized by the genetic results as ALPS patients with FAS mutation (ALPS-FAS) or as ALPS patients with unknown origin (ALPS-U). (A) Double negative T-cell (****P<0.0001); (B) in vitro apoptosis

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functional test (****P<0.0001); (C) sFASL (****P<0.0001); (D) Interleukin-10 levels (P=0.3070). (E) Interleukin-18 (P=0.0514). (F) Evaluation of combinations of laboratory parameters to differentiate ALPS-FAS and ALPS-U.

All data are presented as median ± IQR.

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Tables

Molnár et al. Table 1.

Affected gene	HGVS RNA	HGVS amino acid change	Mechanism	ACMG	Percentage of patients (n=86)	MAF in general population	Provean	SIFT	Meta SVM	Grantham score	HSF (splice)	Previously published in ALPS	Definite ALPS patients	Suspected ALPS patients	Unlikely ALPS diagnosis	Non evaluable	Study conclusion
FAS	c.76C>T	p.Gln26Ter	nonsense	pathogenic	1,16%	0	-	-	-	-	-	NO	1	0	0	0	pathogenic
FAS	c.219C>A	p.Cys73Ter	nonsense	pathogenic	1,16%	0	-	-	-	-	-	YES ALPS[1,2]	1	0	0	0	pathogenic
FAS	c.715_721delGTCATGA	p.Val239HisfsTer2	frameshift	pathogenic	1,16%	0	-	-	-	-	-	NO	1	0	0	0	pathogenic
FAS	c.719_722delinsAGTTA	p.Met240LysfsTer7	frameshift	pathogenic	1,16%	0	-	-	-	-	-	NO	1	0	0	0	pathogenic
FAS	c.742T>G	p.Phe248Val	missense	likely pathogenic	3,49%	0	Damaging	Damaging	Damaging	50	-	NO	3	0	0	0	pathogenic
FAS	c.749G>A	p.Arg250Gln	missense	likely pathogenic	5,81%	0	Damaging	Damaging	Damaging	43		YES ALPS[2,3]	3	1	0	1	pathogenic
FAS	c.776T>G	p.Ile259Arg	missense	likely pathogenic	1,163%	0	Damaging	Damaging	Damaging	97		YES ALPS[4]	0	0	0	1	pathogenic
FAS	c.794A>G	p.Asp265Gly	missense	likely pathogenic	1,16%	0	Damaging	Damaging	Damaging	94		NO	1	0	0	0	pathogenic
FAS	c.826C>A	p.Gln276Lys	missense	likely pathogenic	2,33%	0	Neutral	Tolerated/ Damaging	Damaging	53		NO	1	1	0	0	pathogenic
FAS	c.335-9_335-6delATTT	intronic	splice possible	VUS	1,16%	0	-	-	-	-	Alteration of the WT acceptor site	NO	1	0	0	0	pathogenic
FAS	c.136A>C	p.Thr46Pro	missense	VUS	1,16%	0,00003	Neutral	Tolerated	Tolerated	38	Potential	NO	0	0	1	0	VUS, benign
FAS	c.642T>C	p.Thr214=	synonymous SNP	benign	3,49%	0,766	-	-	-	-	no impact	not applicable	2	1	0	0	benign
FAS	c.196+176C>T	intronic	intronic SNP	benign	1,16%	0,394	-	-	-	-	no impact	not applicable	0	1	0	0	benign
FAS	c.334+46C>T	intronic	intronic SNP	benign	1,16%	0,165	-	-	-	-	no impact	not applicable	1[t]	0	0	0	benign
FAS	c.505+82C>G	intronic	intronic SNP	benign	1,16%	0,387	-	-	-	-	no impact	not applicable	0	1	0	0	benign
FAS	c.506-71C>G	intronic	intronic SNP	benign	1,16%	0,387	-	-	-	-	no impact	not applicable	0	1	0	0	benign
FAS	c.677-95T>C	intronic	intronic SNP	benign	1,16%	0,0392	-	-	-	-	-	not applicable	1	0	0	0	benign
CASP10	c.1216A>T	p.Ile406Leu	missense	likely benign	4,65%	0,00456	Neutral	Tolerated	Tolerated	5	Potential	YES ALPS	1	1	0	2	*VUS*
CASP10	c.1228G>A	p.Val410Ile	missense	benign	1,16%	0,0445	Neutral	Tolerated	Tolerated	29	n/a	YES controversial	0	1	0	0	*likely benign*
CASP10	c.295A>G	p.Lys99Glu	missense	likely benign	1,16%	0,00039	Neutral, Damaging	Tolerated, Damaging	Tolerated	56	Potential	NO	1	0	0	0	*VUS*

Table 1: Genetic variants in FAS and CASP10 genes identified in the patient cohort.

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Genetic variants (coding sequence and protein changes) are listed according to the recommendations of the Human Genome Variation Society (HGVS) nomenclature. Classification of the American College of Medical Genetics and Genomics (ACMG) are shown according to populational frequencies, previous literature data and in silico predictions such as Provean, SIFT, MetaSVM.

Abbreviations: MAF: minor allele frequency, HSF: Human Splicing Finder web-based splice site prediction.

Molnár et al Table 2

Patient no.	Gender	Age	Gene	HGVS RNA	HGVS amino acid change	Zygosity	ACMG	Lympho-proliferation	Cytopenias	Apoptosis assay	DNT	sFASL (pg/ml)	IgG (g/L)	IL10 (pg/ml)	IL18 (pg/ml)	B12 (pg/ml)	Fullfilled criteria (major+minor)*	ALPS classification
39	M	3y	FAS	c.76C>T	p.Gln26Ter	het	pathogenic	Yes	A, N	3,5	7,5 %	>1000	18,5	n/a	2853	n/a	2+4	definite ALPS
107	F	11 mo	FAS	c.715_721 del	p.Val239HisfsTer2	het	pathogenic	Yes	T, A, N	0,4	8,9 %	>1000	38,7	n/a	n/a	1406	3+3	definite ALPS
49	F	14y	FAS	c.719_722delinsAGTTA	p.Met240LysfsTer7	het	pathogenic	Yes	A	1,0	11,0 %	>1000	n/a	119	1229	n/a	3+3	definite ALPS
26	F	49y	FAS	c.742T>G	p.Phe248Val	het	likely pathogenic	n/a	n/a	1,4	5,5 %	129	n/a	n/a	265	n/a	2+0	definite ALPS
28	F	77y	FAS	c.742T>G	p.Phe248Val	het	likely pathogenic	Yes	n/a	1,7	10,5 %	131	n/a	20	446	n/a	3+0	definite ALPS
44	M	4y	FAS	c.742T>G	p.Phe248Val	het	likely pathogenic	Yes	A	1,5	5.7 %	1000	15,4	n/a	1380	n/a	3+2	definite ALPS
30	M	15y	FAS	c.794A>G	p.Asp265Gly	het	likely pathogenic	Yes	T, A, N	1,4	23,0 %	n/a	4,7	n/a	n/a	n/a	3+1	definite ALPS
199	M	6mo	FAS	c.826C>A	p.Gln276Lys	het	likely pathogenic	Yes	n/a	1,4	n/a	312	n/a	n/a	n/a	n/a	2+1	definite ALPS
200	F	6y	FAS	c.826C>A	p.Gln276Lys	het	likely pathogenic	Yes	n/a	n/a	n/a	939	n/a	n/a	n/a	n/a	1+1	suspected ALPS
47	M	7y	FAS	c.335-9_335-6delATTT	intronic	het	VUS	Yes	A	2,8	13,0 %	1000	24,9	n/a	n/a	n/a	3+2	definite ALPS
89	F	2y	FAS	c.136A>C	p.Thr46Pro	het	VUS	Yes	A	9,9	1,3 %	178	9,6	n/a	n/a	n/a	1+0	unlikely ALPS
91	F	12 mo	CASP10	c.295A>G	p.Lys99Glu	het	likely benign	Yes	A	3,0	3,6 %	169	9,0	n/a	n/a	899	3+0	definite ALPS

Table 2: Clinical and laboratory finding of ALPS patients harbouring novel genetic variations

Comments: Fullfilled criteria (major+minor)* are shown without the inclusion of genetic mutation as a major criterion.

Abbreviations: A: anaemia; ACMG: American College of Medical Genetics and Genomics; ALPS: autoimmune lymphoproliferative syndrome; B12: Vitamin B12;

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DNT: double negative T-cell; F: Female; IgG: immunoglobulin G; IL10: interleukin-10; IL18: interleukin-18; M: Male; N: neutropenia; sFASL: soluble FAS-ligand; T: thrombocytopenia.

Table 3. Molnár et al.

Patient no.	Gender	Age	Gene	HGVS RNA	HGVS amino acid change	Zygosity	ACMG	Lympho-proliferation	Cytopenias	Apoptosis assay	DNT	sFASL (pg/ml)	IgG (g/L)	IL10 (pg/ml)	IL18 (pg/ml)	B12 (pg/ml)	ALPS classification
95	F	10mo	IKBKG	c.549G>C	p.Gln183His	homo	Likely Pathogenic	Yes	A	2.7	2.1	n/a	14.3	n/a	n/a	n/a	definite ALPS
			ORAI1	c.144_150 dup	p.Thr53ArgfsTer39	het	Uncertain Significance										
2	F	14y	MYO5B	c.154A>G	p.Ile52Val	n/a	Uncertain Significance	Yes	T,A,N	2.1	1.8	146.9	n/a	28.8	436.4	n/a	definite ALPS
42	F	4y	PRF1	c.539+83C>T	*intronic*	het	Uncertain Significance	Yes	A	1.7	5.1	>1000	15.3	n/a	3238	n/a	definite ALPS
				c.272C>T	p.Ala91Val	het	Uncertain Significance										
45	M	24y	PRF1	c.755A>G	p.Asn252Ser	n/a	Uncertain Significance	Yes	n/a	1.6	n/a	297.4	n/a	74.2	2269.8	n/a	definite ALPS
36	F	18mo	PRF1	c.948C>A	p.Phe316Leu	n/a	Likely Pathogenic	Yes	T,A,N	2.9	2.9	486	11.05	82	1931	>1000	definite ALPS
38	F	15y	RAG1	c.2290C>T	p.Arg764Cys	homo	Likely Pathogenic	Yes	T,A,N	n/a	5.5	391.8	n/a	118,3	2189.6	n/a	definite ALPS
1	M	9y	STAT3	c.2144C>T	p.Pro715Leu	n/a	Pathogenic	Yes	T, N	2.3	2.7	175	n/a	38	455	n/a	suspected ALPS
105	F	16mo	STAT3	c.2144C>T	p.Pro715Leu	n/a	Pathogenic	Yes	A, N	9.4	2.6	154	14.1	n/a	n/a	549	suspected ALPS
119	M	14y	TNFRSF13B	c.310T>C	p.Cys104Arg	n/a	Uncertain Significance	Yes	T, A, N	4.6	2.3	48	4.63	n/a	n/a	n/a	suspected ALPS
62	M	4y	TNFRSF13B	c.310T>C	p.Cys104Arg	het	Uncertain Significance	Yes	A, N	6.8	2	181	5.77	n/a	n/a	n/a	suspected ALPS

Table 3: Clinical and laboratory findings of ALPS-U patients with genetic mutations

Abbreviations: A: anaemia; ACMG: American College of Medical Genetics and Genomics; ALPS: autoimmune lymphoproliferative syndrome; B12: Vitamin B12; DNT: double negative T-cell; F: Female; IgG: immunoglobulin G; IL10: interleukin-10; IL18: interleukin-18; M: Male; N: neutropenia; sFASL: soluble FAS-ligand; T: thrombocytopenia.

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Figure 1. Molnár et al.

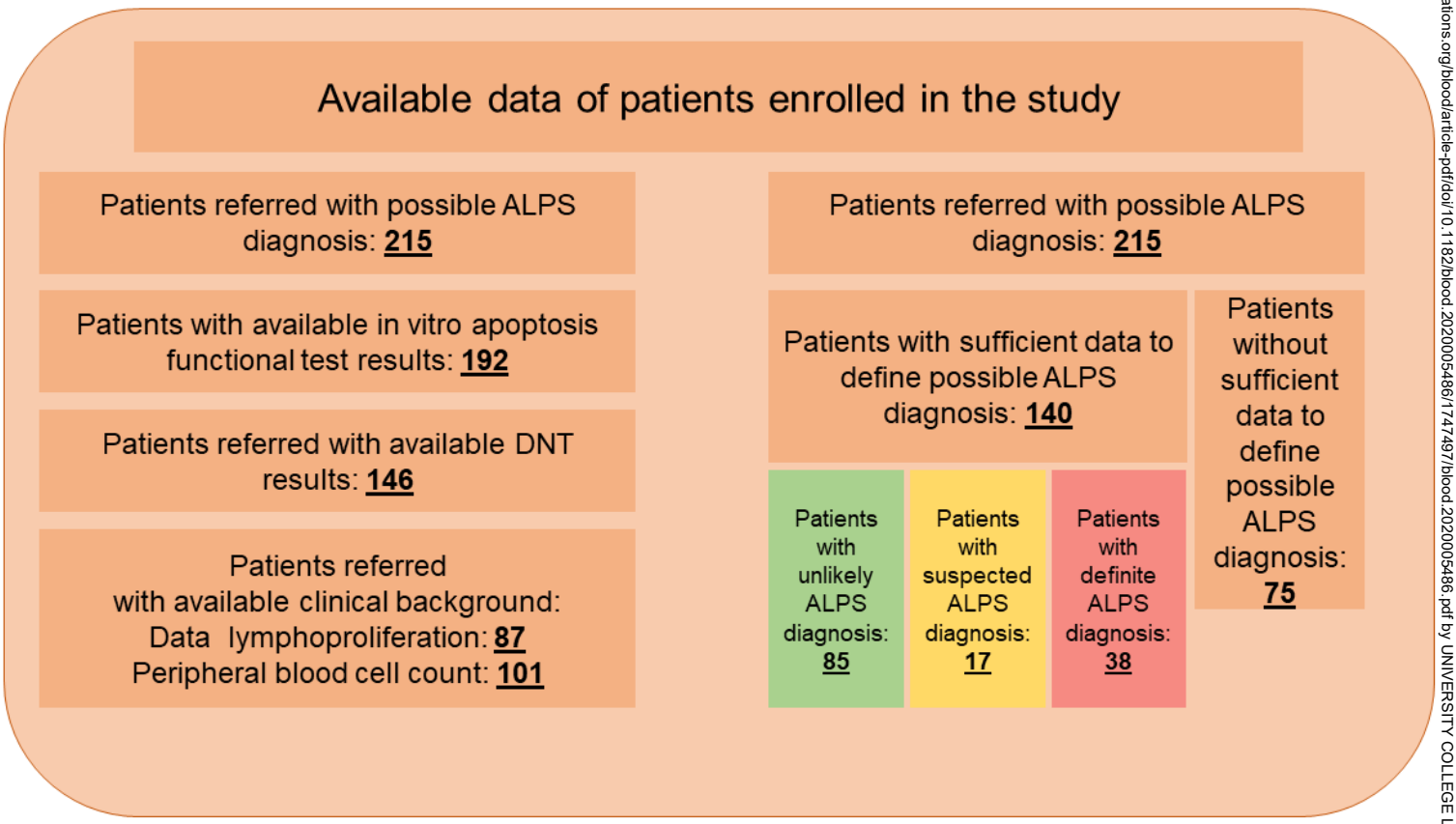

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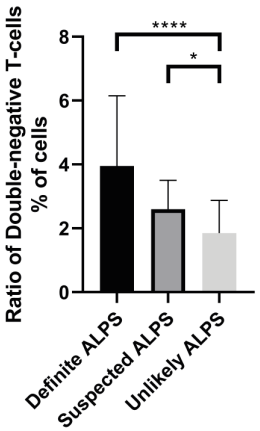
Figure 2. Molnár et al.

A

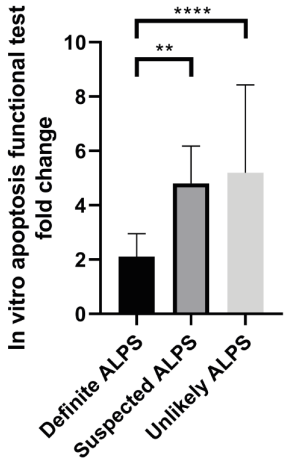
	Definite ALPS	Suspected ALPS	Unlikely ALPS
Lymphoproliferation/ lymphadenopathy/ splenomegaly	33/34 (97.1%)	14/16 (87.5%)	22/40 (55%)
	Definite ALPS	**Suspected ALPS**	**Unlikely ALPS**
Multilineage cytopenia	20/29 (69%)	9/16 (56.3%)	30/53 (56.6%)

B



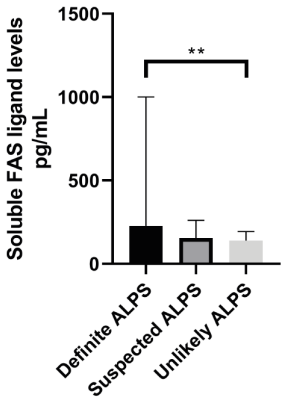
	Definite ALPS	Suspected ALPS	Unlikely ALPS
Normal	0/34 (0%)	0/14 (0%)	33/68 (48.5%)
Abnormal	34/34 (100%)	14/14 (100%)	35/68 (51.5%)
Median (Range)	3.95% (1.8% - 23%)	2.6% (1.9% - 6.7%)	1.85% (0% - 13.9%)

C



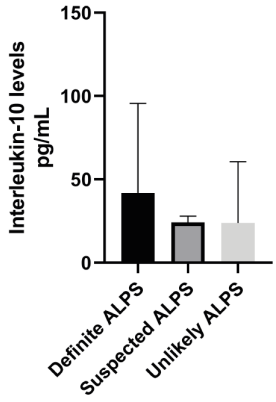
	Definite ALPS	Suspected ALPS	Unlikely ALPS
Normal	7/33 (21.2%)	12/16 (75%)	69/80 (86.3%)
Equivocal	11/33 (33.3%)	2/16 (12.5%)	8/80 (10%)
Abnormal	15/33 (45.5%)	2/16 (12.5%)	3/80 (3.8%)
Median (Range)	2.1 (0.4 - 6.7)	4.8 (1.3 - 18.1)	5.2 (1.1 - 30.5)

D



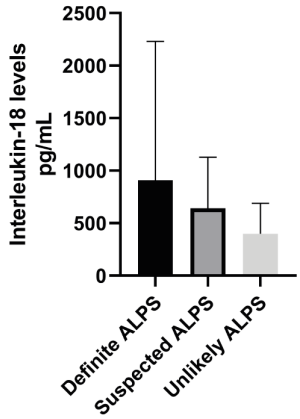
	Definite ALPS	Suspected ALPS	Unlikely ALPS
Normal	18/35 (51.4%)	8/11 (72.7%)	46/60 (76.7%)
Abnormal	17/35 (48.6%)	3/11 (27.3%)	14/60 (23.3%)
Median (Range) (pg/ml)	195 (44 - >1000)	154 (23.5 - 939)	139 (35 - >1000)

E



	Definite ALPS	Suspected ALPS	Unlikely ALPS
Normal	9/18 (50%)	2/2 (100%)	5/6 (83.3%)
Abnormal	9/18 (50%)	0/2 (0%)	1/6 (16.7%)
Median (Range) (pg/ml)	41.75 (19 - 169.2)	24.2 (20.5 - 27.9)	23.8 (8 - 137.6)

F



	Definite ALPS	Suspected ALPS	Unlikely ALPS
Normal	7/21 (33.3%)	1/2 (50%)	5/9 (55.6%)
Abnormal	14/21 (66.6%)	1/2 (50%)	4/9 (44.4%)
Median (Range) (pg/ml)	908.9 (265 - 3255)	642.1 (157 - 1127.2)	398.2 (206 - 1375.2)

G

	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
DNT & In vitro apoptosis functional test	79.3%	93.7%	85.2%	90.8%
DNT & sFASL	41.9%	87.2%	68.4%	69.5%
In vitro apoptosis functional test & sFASL	36.7%	96.4%	84.6%	73.6%

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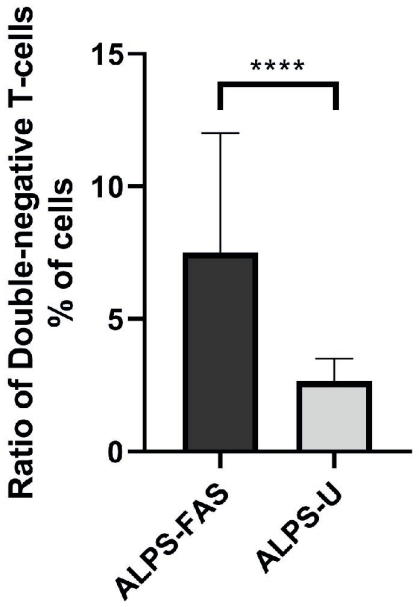
Figure 3. Molnár et al.

A



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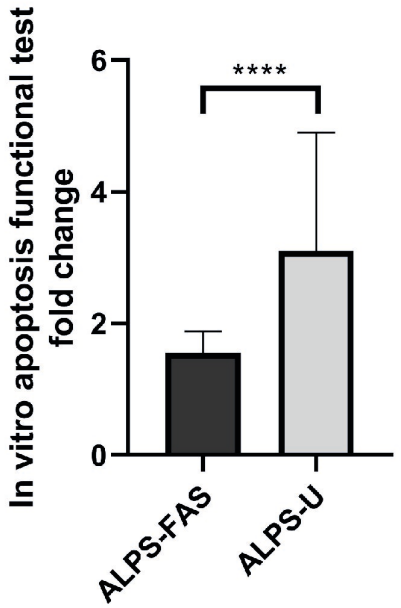

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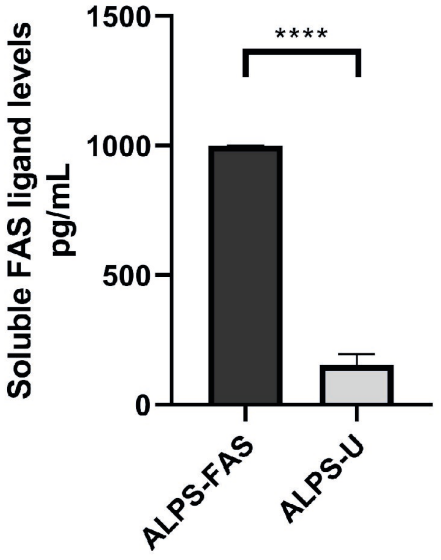

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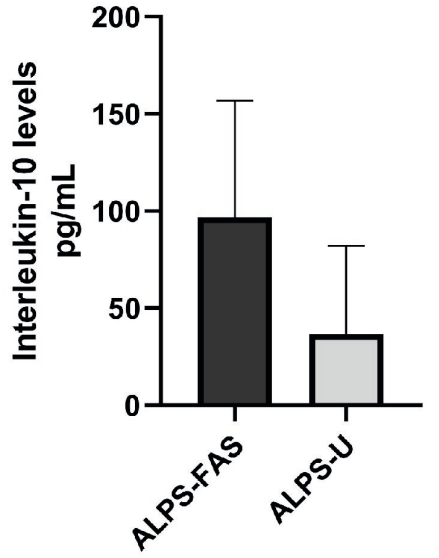

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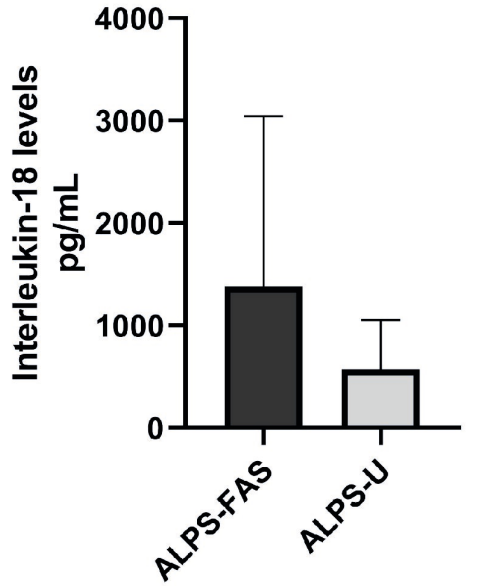

F

	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
DNT & In vitro apoptosis functional test	90.9%	51.7%	41.7%	93.8%
DNT & aFASL	83.3%	81.5%	66.7%	91.7%
In vitro apoptosis functional test & sFASL	76.9%	92.0%	83.3%	88.5%

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