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EVALUATION OF DIAGNOSTIC MARKERS OF AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME

PhD thesis

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List of Abbreviations

ACMG – American College of Medical Genetics and Genomics

AICD – activation of 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

HGVS – Human Genome Variation Society

HSF – Human Splicing Finder

IgG – Immunoglobulin G

MAF – Minor allele frequency

MCD – mixed connective tissue disease

mTOR – mammalian target of rapamycin

NGS – next generation sequencing

NIH – National Institute of Health

PPV – positive predictive value

sFASL – soluble FAS ligand

SNP – single nucleotide polymorphism

TIGER – targeted immunodeficiency and gastrointestinal enrichment panel

VUS – variants of unknown significance

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

1. INTRODUCTION

1.1 Pathogenesis

Autoimmune lymphoproliferative syndrome (ALPS or Canale-Smith syndrome) is a rare immunodeficiency caused by abnormal extrinsic FAS-mediated apoptotic pathway.[1-3] FAS-mediated apoptosis plays an important role in programmed cell death of (i) virus-infected cells induced by cytotoxic T-cells; (ii) T-cells after immune response; (iii) cells participating in inflammation and in immune cell regulation preventing autoimmunity. Non-malignant, chronic lymphoproliferation, unexplained lymphadenopathy, hepatosplenomegaly; immune-mediated cytopenia; hypergammaglobulinemia (IgG) are all pathognomic hallmarks of non-functional extrinsic apoptotic pathway.[3] Defective lymphocyte apoptosis could principally cause autoimmune manifestations, mainly multilineage cytopenia and less frequently nephritis, hepatitis, uveitis, arthritis or colitis. These symptoms show similarity to malignant, infectious and other lymphoproliferative diseases. ALPS can be distinguished by histopathology with a unique expression of CD45RO in the lymph node paracortical $CD3^{+}TCR\alpha\beta^{+}$ $CD4^{-}CD8^{-}$ double negative T-cell (DNT) population. The extrinsic apoptotic pathway contains Fas ligand (FasL) belonging to the tumour necrosis family, which induce apoptosis of various Fas receptor bearing cells binding its receptor Fas. In activated T cells, this pathway is involved and responsible for the down-regulation of T cell-mediated cytotoxicity as well as immune reactions.[4] Furthermore, patients with ALPS and also genetically affected siblings without 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.[5-7]

1.2 Genetic background and classification

In ALPS, the defective extrinsic apoptotic signalling is caused by germline and somatic mutations in *FAS* (*TNFRSF6, CD95, APO1*), *FASLG,* and *CASP10* genes, encoding Fas cell surface death receptor, FAS ligand and caspase 10 proteins respectively.[8] The majority of ALPS patients (almost 70 %) present with germline heterozygous or somatically acquired *FAS* mutations. Rarely *FAS* mutations are inherited

in an autosomal recessive manner.[2, 3, 9, 10] *FAS* gene encodes a protein receptor expressed on B and T lymphocyte lineages. ALPS patients predominantly present with defective apoptotic pathway in T lymphocytes and rarely in B lymphocytes. In contrast, patients with *CASP8* mutations have mainly apoptotic defect in B and T cells, as well as NK lymphocyte lineage. Therefore, different disease should be considered in patients with *CASP8* mutations; however, previously these patients were also considered to have clinically ALPS, because caspase 10 and 8 have almost similar functions in the caspase cascade and patients with *CASP8* and *CASP10* mutations show closely similar clinical symptoms.[11]

On the basis of the underlying pathogenic genetic variants, ALPS is classified into sub-categories such as type I-III.[2, 8] ALPS type Ia is based on heterozygous mutations of the *FAS* gene. The type Ib is depicted as mutation in *FASLG* gene. Those patients who have mutations in the caspase family, namely in the *CASP10* gene are defined as ALPS type II. In ALPS patients without defined mutation in the above-mentioned genes (20-30 % of the cases), the condition is classified as ALPS type III. [2, 7, 9, 12-18]

Until 2007, all ALPS clinical manifestations were considered to be related to genes that impair the Fas-mediated extrinsic apoptotic pathway. However, the first ALPS-like patient was described by Oliveira et al.[19], who presented normal Fas-mediated apoptosis, abnormal lymphocyte apoptosis caused by abnormal intrinsic apoptotic pathway associated a gain of function mutation in *NRAS.[18]*

For simplicity, the novel ALPS classifications of Oliveira *et al.* in 2010 renamed the ALPS subtypes according to the name of affected genes. Therefore, patients with ALPS phenotype and having germline homozygous or heterozygous *FAS* mutations were classified as ALPS-FAS. Also, patients presenting somatic mutation in *FAS* gene were defined as ALPS-sFAS. Mutation in FASL gene were categorised into ALPS-FASL group. Similarly, the group of patients with *CASP10* mutation was unified under ALPS-CASP10[20]. Those patients, who clinically fulfil the ALPS diagnostic criteria, but do not have any mutation in the extrinsic apoptotic pathway, were replaced in the ALPS-U (undetermined) group.

1.3 Laboratory biomarkers

Impaired activation of induced cell death (AICD) in patients suffering from ALPS results in developing an abnormal T cell repertoire.[21] The accumulation of autoreactive $CD3^{+}TCR\alpha\beta^{+}$ $CD4^{-}CD8^{-}$ double negative T-cells (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.[22, 23] The origin of DNT population is still not fully known. Some data suggests that DNTs are thymocyte-derived regulatory T cells (T_{reg})[24] while others theorise that these cells originate from $CD8^{+}$ T cells that lost CD8 expression; however, some studies do not support this theory.[25, 26] The DNTs resemble normally differentiated T-cells and usually express naive 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.[16, 27] Mildly elevated DNT was previously reported in a variety of autoimmune diseases, such as systemic lupus erythematosus and immune thrombocytopenic purpura; while marked elevation of DNT (5% or above) was only described in ALPS patients. Of note, patients with lymphocytopenia may present a false negative DNT due to abnormalities of peripheral lymphocyte count.

The second main diagnostic biomarker for ALPS is the defect in the FAS-mediated apoptosis pathway, which can be monitored by the FAS-mediated apoptosis functional assay in the laboratory environment. Separated peripheral blood mononuclear cells (PBMC) from patients' blood samples are stimulated and cultured for six to seven days with anti-CD3 and IL-2. After incubation, cells are treated with anti-FAS antibody. In healthy cells, this stimulus trigger apoptosis, while in cells derived from ALPS patients the apoptosis rate is low due to the defect of the FAS-mediated apoptosis pathway. The apoptosis rate is monitored by staining against apoptosis specific proteins, and apoptotic cells are enumerated by flow-cytometry. Of note, DNTs are not viable in cell cultures, therefore FAS-mediated in vitro apoptosis functional assay is normal in patients with somatic *FAS* mutations. Additionally, patients with defective FAS ligand have normal FAS-mediated in vitro apoptosis results, because the test utilizes an external substitute to FAS ligand, and in these patients the downstream cascade is unaffected. The FAS-

mediated pathway can be also affected in other autoimmune diseases, such as multiple sclerosis, type I diabetes mellitus and systemic lupus erythematosus.

Defective FAS-mediated apoptosis pathway usually causes an upregulation of soluble FAS ligand, therefore in ALPS patients the elevation of soluble FAS ligand in the serum can be observed as a biomarker of ALPS.[28] Other consequential, non-specific laboratory features of ALPS are raised levels of interleukin-10 and -18 (IL-10 and IL-18), elevated levels of serum B12 and hypergammaglobulinemia. The appearance of autoantibodies, including Direct Antibody Test positivity, anti-platelet antibodies and anti-neutrophil antibodies, is a frequently observed feature of ALPS.

The sensitivity and specificity of the combinations of these biomarkers have been previously scarcely studied.[29, 30]

1.4 Interpretation of available diagnostic protocols

The original ALPS classification released by National Institute of Health ALPS group (NIH-ALPS) defined three elementary criteria, which need to be present to establish the diagnosis of ALPS. According to this protocol non-malignant, chronic lymphoproliferation needs to be present with elevated DNT accompanied by in vitro evidence of defective Fas-mediated apoptosis. Mutations in the Fas-mediated pathway genes (*FAS*, *FASLG*, *CASP10)* were only supportive, but not diagnostic evidence.[31]

In 2008, the above-mentioned diagnostic protocol was extended by identifiable germline or somatic mutation in *FAS*, *FASLG*, *CASP10*, *NRAS* genes, as a major criterion and also, several minor criteria were added such as, autoimmune cytopenias, moderate elevation in DNT, elevated serum IgG, IL-10, vitamin B12, plasma Fas ligand level. According to this extended classification system three major, or two major plus two minor criteria are needed to be present at the same time to set up the diagnosis of ALPS[32].

In 2010, the diagnostic criteria for ALPS were further revised. Two required criteria were defined, such as chronic (more than 6 months) non-malignant, non-infectious lymphadenopathy or splenomegaly and elevated DNT level present at the same time. Also, two primary and four secondary accessory criteria were added. The defective lymphocyte apoptosis and somatic or germline pathogenic mutation in *FAS*, *FASLG*, *CASP10* were mentioned as primary and elevated plasma sFASL levels, typical immunohistological findings as reviewed by an experienced hematopathologist,

autoimmune cytopenias, elevated IgG levels and family history of non-malignant / non-infectious lymphoproliferation with or without autoimmunity were mentioned as secondary accessory criteria. According to this classification, both required symptoms and one primary accessory criterion should be present to set up definitive ALPS diagnosis. In addition, a probable ALPS diagnosis is based on the completed two required criteria plus one secondary accessory criterion.[2]

In 2019, this complicated classification system was simplified by the European Society for Immunodeficiencies (ESID), where five major criteria, such as splenomegaly, lymphadenopathy (more than 3 months, more than 3 nodes, non-infectious, non-malignant), autoimmune cytopenias (at least in two lineages) , history of lymphoma and affected family member were defined and five additional elevated biomarkers were named, like sFASL, IL-10, vitamin B12, impaired Fas-mediated apoptosis and increased level of DNTs. According to this clinical classification one major and at least one additional criterion are needed to be present at the same time to fulfil the requirements of ALPS diagnosis.

2. OBJECTIVES

In this study we aimed to collect and evaluate data of patients with ALPS. The first objective was to collect and analyse the population referred between 2008 - 2018 with potential ALPS to the Laboratory of Immunology of Great Ormond Street Hospital. As clinical signs and symptoms characteristic for ALPS share clinical features with a great variety of other diseases, not all referred patients had ALPS. By characterizing this population, the results can provide an overview of similarities of ALPS-like diseases. We aimed to collect and analyse the clinical symptoms of ALPS as well as the available laboratory findings that can further improve the diagnosis. As the records of some patients were found incomplete in retrospective data collection, we planned to establish a patient group that could potentially had ALPS but not enough diagnostic criteria were available, this group is described as suspected ALPS population. We evaluated the frequency of the clinical symptoms in patients with definite or suspected ALPS, as well as in patients with unlikely ALPS.

We also aimed to evaluate the specificity and sensitivity of different laboratory biomarkers in patients with ALPS. As the diagnostic criteria of this disease relies on multiple symptoms and laboratory results, including molecular genetic screening, the final diagnosis of patients is an elaborate and time-consuming process. Furthermore, some of the biomarkers needed for diagnosis are costly and can take weeks to receive results. Thus, by characterizing the biomarker combinations with the highest sensitivity and specificity, we aim to provide an improved diagnostic guideline, which could improve the diagnostic process.

As ALPS is a polygenic disease, we hypothesized that different forms might have distinct patterns in diagnostic criteria. We aimed to evaluate the available molecular genetic results to determine the disease-causing potency of the different genetic variants. Furthermore, we aimed to categorize patients with definite or suspected ALPS into groups with or without damaging mutation in the FAS gene. We aimed to characterize and compare the recorded symptoms and laboratory findings in the distinct genetic groups. Furthermore, we aimed to provide an extension of the diagnostic criteria to underline the laboratory findings that are more frequent in patients with damaging FAS mutations.

3. RESULTS

3.1 Description of clinical and laboratory parameters

Out of the 215 patients referred with clinical evidence of ALPS diagnosis, clinical background was recorded for 87 patients including lymphoproliferation, lymphadenopathy, splenomegaly. Haemoglobin, platelet and white blood cell counts were available for 101 patients. Apoptosis functional test was performed for 192, DNT for 146 cases, 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. The working definitions of ESID was applied for definite ALPS, where elevated DNT was 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. Cases lacking information about at least two major diagnostic and at least one minor criterion were defined as non-evaluable (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). Another 17 patients (12.1%)

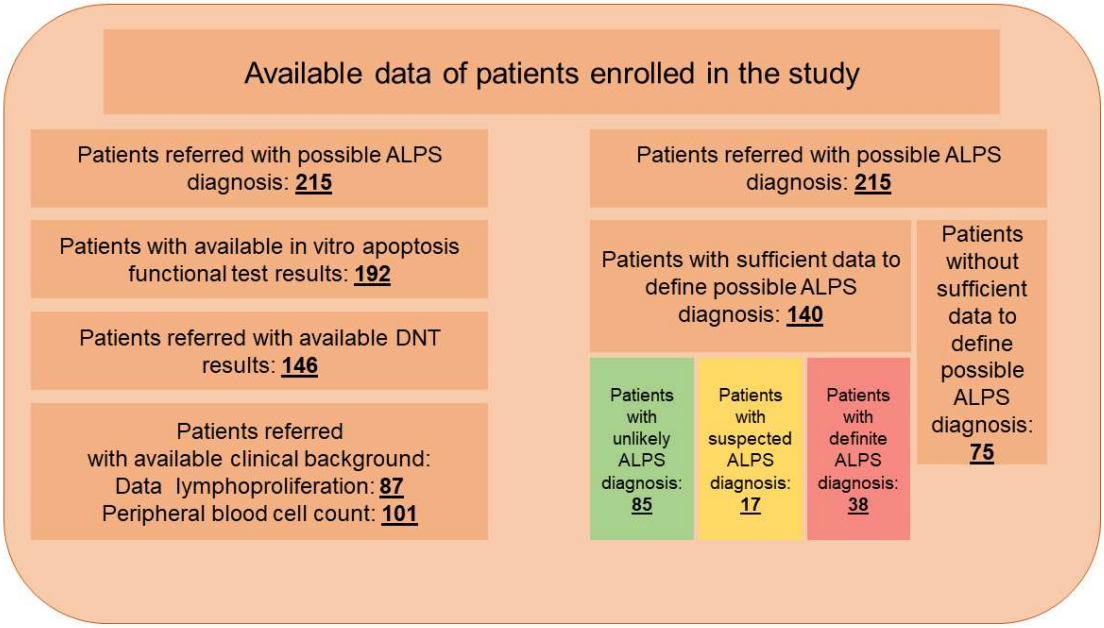

Figure 1.: Summary of the data available of the patients enrolled in this study. 215 patients were referred with potential ALPS

fulfilled the criteria of suspected ALPS (median age at first referral: 13.1 years, range: 1 month-19 years), while 85 patients (60.7%) did not meet ALPS diagnostic criteria (unlikely ALPS group, median age at first referral: 10.3 years, range: 2 month-64 years).

he 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, Table 1). Outcome data was available in a limited number of patients. Out of 34 definite ALPS patients 3 (3/34, 8.82%) and another 3 suspected ALPS patients (3/16; 19.75%) underwent splenectomy. Lymphoma development was described in two (2/34; 5.88%) definite ALPS patients.

Table 1.: Clinical data of the enrolled patients: lymphoproliferation (P<0.0001 definite vs unlikely ALPS) and multilineage cytopenia (P=0.5168)

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

Abnormally high DNT was defined as higher than 1.8% of the $\alpha\beta^+$ T cell population and 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 2).

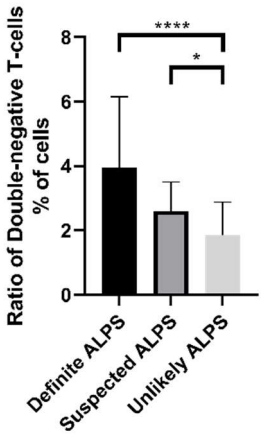

Figure 2.: Double-negative T-cell percentages in the different patient groups

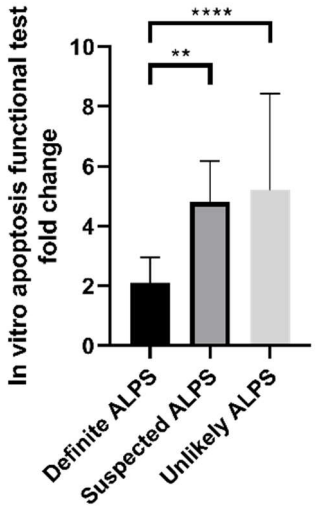

Figure 3.: Changes in the in vitro apoptosis functional test in different patient groups

Regarding in vitro FAS-mediated apoptosis function assay, more than three-fold change in the number of apoptotic cells was considered as normal, defective apoptosis was defined as lower than 2.0-fold change. The range between 2.0-3.0 was described as equivocal. In definite ALPS (n=33), 15 patients had abnormal apoptosis functional test (45.5%), 11 had equivocal (33.3%) and 7 had normal results. While in suspected ALPS (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 (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 3).

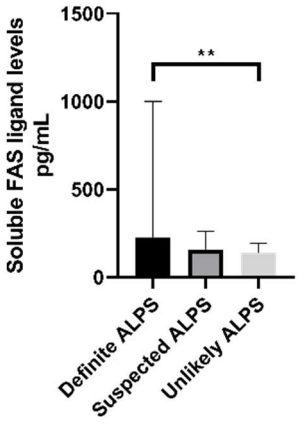

Figure 4.: Differences in soluble FAS ligand levels in the different patient groups

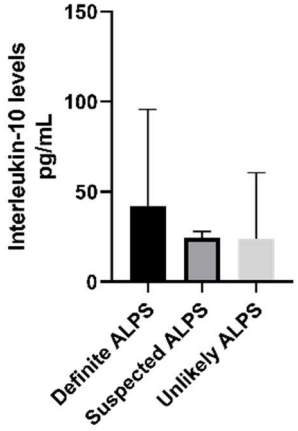

Figure 5.: Differences in Interleukin-10 levels in the different patient groups

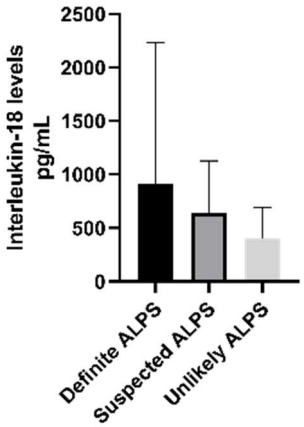

Figure 6.: Differences in Interleukin-18 levels in the different patient groups

Normal sFasL level was defined as: 200 pg/ml or less. The sFASL level was higher in definite compared to unlikely ALPS (**P=0.0013; definite ALPS: median 195, range:

44->1000 pg/ml; suspected ALPS: median 154, 24-939 pg/ml; unlikely ALPS: median 139, range: 35->1000 pg/ml, Figure 4).

The normal value of IL-10 was defined as lower than 40 pg/ml. IL-10 level showed tendency of being elevated in definite ALPS (median: 42, range: 19-169 pg/ml) compared to suspected ALPS (median: 24, range: 21-28 pg/ml) and unlikely ALPS (median: 24, range: 8-138 pg/ml, P=0.0624; Figure 5). The normal range of IL-18 is lower than 500 pg/ml. 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, range: 157-1127 pg/ml, unlikely ALPS: median 398, range: 206-1375 pg/ml, P=0.0615; Figure 6).

3.2 Analysis of biomarker combinations in the diagnostic process

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 (Table 2). 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; 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; the sensitivity of this combination was 36.7% and the specificity was 96.4%. All three combinations discriminated the definite and the unlikely ALPS groups. (****P<0.0001 for DNT and in vitro apoptosis functional test;

Table 2.: Summary of the specificity and sensitivity of different biomarker combinations for ALPS.

	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%

**P=0.0040 for DNT and sFASL; ***P=0.00012 for in vitro apoptosis functional test and sFASL).

3.3 Genetic results

Genetic results were available for 87 patients. Variants were described according to the Human Genome Variation Society (HGVS) nomenclature. We considered pathogenic, likely pathogenic and variants of unknown significance (VUS), classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines as genetic variants that possibly alter function (Table 3). 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).[5, 33]

Table 3.: Genetic variants in FAS and CASP10 genes identified in the patient cohort.

Affected gene	HGVS RNA	HGVS amino acid change	Mechanism	ACMG	Patients, % (n=86)	MAF in general population	Provean	SIFT	MetaSVM	Grantham score	HSF (splice)	Previously published in ALPS?	Diagnosis			Non evaluable	Study conclusion
													Definite	Suspected	Unlikely		
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	1,16%	0	Damaging	Damaging	Damaging	97		YES ALPS [5]	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	NA	2	1	0	0	benign
FAS	c.196+176C>T	intronic	intronic SNP	Benign	1,16%	0,394	-	-	-	-	no impact	NA	0	1	0	0	benign
FAS	c.334+46C>T	intronic	intronic SNP	Benign	1,16%	0,165	-	-	-	-	no impact	NA	1[t]	0	0	0	benign
FAS	c.505+82C>G	intronic	intronic SNP	Benign	1,16%	0,387	-	-	-	-	no impact	NA	0	1	0	0	benign
FAS	c.506-71C>G	intronic	intronic SNP	Benign	1,16%	0,387	-	-	-	-	no impact	NA	0	1	0	0	benign
FAS	c.677-95T>C	intronic	intronic SNP	Benign	1,16%	0,0392	-	-	-	-	-	NA	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*

ACMG - American College of Medical Genetics and Genomics; HGVS - Human Genome Variation Society; HSF - Human Splicing Finder; SNP – Single nucleotide polymorphism VUS - variants of unknown significance;

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)[34, 35] 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 splice region). The variant lies in the close proximity to an acceptor splice site, the in silico splice prediction (Human Splicing Finder, HSF) suggested that the variant altered splice site, most probably affecting splicing[36].

The following benign *FAS* gene variants were identified. The *FAS* c.136A>C (p.Thr46Pro) was categorised as 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.

Table 4.: Clinical and laboratory finding of ALPS patients harbouring novel genetic variations.

Patient no.	Sex	Age	Gene	HGVS RNA	HGVS amino acid change	Zygosity	ACMG	Lympho-proliferation	Cytopenias	Apoptosis assay	DNTs	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	7,50%	>1000	18,5	n/a	2853	n/a	2+4	definite ALPS
107	F	11mo	FAS	c.715_721 del	p.Val239HisfsTer2	het	pathogenic	Yes	T, A, N	0,4	8,90%	>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	11,00%	>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	5,50%	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	10,50%	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	23,00%	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	13,00%	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	1,30%	178	9,6	n/a	n/a	n/a	1+0	unlikely ALPS
91	F	12mo	CASP10	c.295A>G	p.Lys99Glu	het	likely benign	Yes	A	3	3,60%	169	9	n/a	n/a	899	3+0	definite ALPS

A – Anaemia; ACMG - American College of Medical Genetics and Genomics; F – Female; HGVS - Human Genome Variation Society; M – Male; N – Neutropenia; SNP – Single nucleotide polymorphism; T – Thrombocytopenia; VUS - variants of unknown significance;

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)[37] *CASP10* p.Ile406Leu 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[38-40] 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 4. *FASLG* and *CASP8* pathogenic mutations were not identified in our definite or suspected patient cohort.

Table 5.: Clinical and laboratory findings of ALPS-U patients with genetic mutations

Patient no.	Sex	Age	Gene	HGVS RNA	HGVS amino acid change	Zygosity	ACMG	Lympho-proliferation	Cytopenias	Apoptosis assay	DNTs	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.márc	n/a	n/a	n/a	definite ALPS
			ORAI1	c.144_150dup	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.aug	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.márc	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	febr.74	aug.69	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	05.nov	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	jún.89	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.jan	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

A – Anaemia; ACMG - American College of Medical Genetics and Genomics; HGVS - Human Genome Variation Society; N – Neutropenia; SNP – Single nucleotide polymorphism; T – Thrombocytopenia; VUS - variants of unknown significance;

In our ALPS-U patient group, the targeted immunodeficiency and gastrointestinal enrichment (TIGER) next generation sequencing (NGS) panel for immunodeficiency genes identified pathogenic, likely pathogenic or unknown significant variants in 6 definite and 4 suspected ALPS patients (Table 5). 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.

3.4 Differences in ALPS-FAS and ALPS-U

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 7). 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 8). 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 levels (n=29/35; median 152 pg/ml; range:23.5-486 pg/ml; ****P< 0.0001, Figure 9).

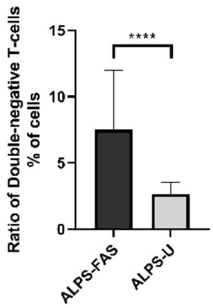

Figure 7.: Comparison of DNT levels of ALPS-FAS and ALPS-U patients

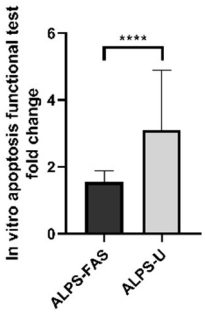

Figure 8.: Comparison of in vitro apoptosis function of ALPS-FAS and ALPS-U patients

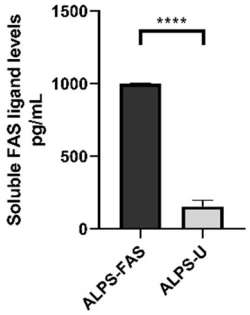

Figure 9.: Comparison of sFASL levels of ALPS-FAS and ALPS-U patients

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 10, 11).

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, Table 6).

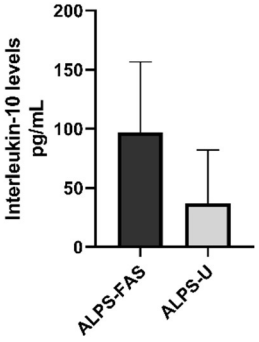

Figure 10.: Comparison of Interleukin-10 levels of ALPS-FAS and ALPS-U patients

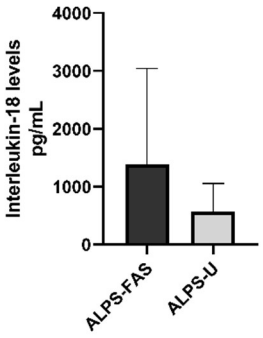

Figure 11.: Comparison of Interleukin-18 levels of ALPS-FAS and ALPS-U patients

Table 6.: Specificity and sensitivity of different biomarker combinations for ALPS-FAS

	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%

4. 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, 31, 32, 41] 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.[41] 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.[29, 42] Furthermore, 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. This group was created to include those patients who have possibly received immunosuppressive treatments at the time of the referral. 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, 41] 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 population similar to other previous publications.[6] Furthermore, all definite and suspected ALPS patients had elevated DNT, when the test was performed.[2, 3, 41] However, 51.5% of patients with unlikely ALPS diagnosis also showed abnormal DNT levels and the specificity (50%) and the positive predictive value (PPV) of DNT (43%) proved to be relatively low as previously published.[30] Several types of immunodeficiencies, such as X-linked immunodeficiency with magnesium defect, Epstein-Barr virus infection and neoplasia (XMEN)[43], autoimmune disorders such as juvenile systemic lupus erythematosus, mixed connective tissue disease (MCD) and

severe infections[44, 45] can also cause elevated DNT.[46] 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. Furthermore, 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 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 population (Figure 3-4.). 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.[47] 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 ACMG 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 ALPS except the apoptosis functional assay. However, patient number 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* gene have a higher penetrance than extracellular mutations.[5, 34] In our study, peripheral blood samples were used for genetic screening, and 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 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.[48] *MYO5B* gene is related to microvillus inclusion disease characterised by gastrointestinal and neurological symptoms, but not related to 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.[49] 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,[50] the same mutation was independently described in Evans syndrome.[49] *TNFRSF13B* c.310T>C a common functional 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.[51] 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.[29, 30, 52] 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%.

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 [39]) 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.[38] No literature has 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 assay and elevated sFASL. However, this tendency was not 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, 53, 54]

Our findings illustrate that both major and minor biomarkers defined in 2010 by classifications of Oliveira et al.[2] 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.[30, 52] 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, [55] while elevated IL-10 was reported as the direct cause for serum lipoprotein-alterations.[56] 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 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. This study was approved by the Bloomsberry Research Ethics Committee and was conducted in accordance with the Declaration of Helsinki.

5. CONCLUSIONS

We have evaluated the clinical and laboratory data of 215 patients referred with potential diagnosis of ALPS. From this cohort, 140 patients had sufficient data for categorisation. We have identified 38 patients with definite and 17 patients with suspected ALPS. Lymphoproliferation was a strong indicator for ALPS, definite ALPS patients had a 97.1% frequency of lymphoproliferation, while patients with unlikely ALPS had 55% frequency. According to our findings, multilineage cytopenia was a less reliable marker of ALPS, it only had a frequency of 69% in definite ALPS patients, and 56.6% in unlikely ALPS patients.

Regarding laboratory findings, our data uncovered that elevated DNT, deficient in vitro apoptosis and elevated soluble FAS ligand levels were all significantly more likely to be found in ALPS patients, compared to patients with unlikely ALPS. Thus, we conclude that DNT levels, in vitro apoptosis assay and soluble FAS ligand levels might be the optimal choice of testing if ALPS is suspected. We found that DNT in combination with in vitro apoptosis functional test had the highest sensitivity, thus, it might be the optimal first choice during ALPS differential diagnosis. We also found that abnormal in vitro apoptosis function in combination with soluble FAS ligand levels had the highest specificity for ALPS. Hence, if ALPS must be first ruled out, this biomarker combination might be the optimal choice.

We evaluated the genetic background of these patients. We identified 11 variants of the *FAS* gene with a potency of affecting protein function, out of which 8 novel variants not reported as an underlying mutation of ALPS previously. Evaluating the laboratory parameters of patients with or without identified *FAS* mutations, we found that elevated DNT, altered in vitro apoptosis and elevated soluble FAS ligand were more common in ALPS patients with *FAS* mutation compared to ALPS patients with an undetermined genetic background.

In conclusion our results support the efficacy of the currently available diagnostic criteria and help in the optimisation of the diagnostic algorithm of ALPS in the clinical practice.

6. SUMMARY

Autoimmune lymphoproliferative syndrome (ALPS) is a rare immunodeficiency, that appears with non-malignant, chronic lymphoproliferation in the clinical practice. Main laboratory results in this condition include multilineage cytopenia, hypergammaglobulinemia (IgG), elevated levels of interleukin-10, interleukin-18, soluble FAS ligand and vitamin B12. Other, more specific laboratory findings in this condition include elevated double negative T-cell percentage and the deterioration of the in vitro apoptosis functional test. Currently accepted diagnostic protocols rely on these clinical and laboratory findings. ALPS is caused by damaging genetic mutations in the extrinsic apoptotic pathway, including genes *FAS*, *FASLG* and *CASP10*.

Our aim was to evaluate the available clinical and laboratory data of patients referred with ALPS to the Laboratory of Immunology of Great Ormond Street Hospital between 2008-2018.

Altogether, 215 patients were referred with potential ALPS diagnosis, from which 140 patients had sufficient data for evaluation (38 patients with definite ALPS, 17 suspected ALPS, while ALPS could be ruled out in 85 patients). In the patient cohort with definite ALPS lymphoproliferation was significantly more frequent than in patients with unlikely ALPS. Furthermore, defective in vitro apoptosis, elevated DNT and soluble FAS ligand levels were also differed between these groups. We evaluated the sensitivity and specificity of different biomarker combinations and found that elevated DNT in combination with defective in vitro apoptosis were the most sensitive, while defective in vitro apoptosis with elevated soluble FAS ligand combination had the highest specificity. We evaluated the genetic background in the context of biomarkers. We found that elevated DNT, defective in vitro apoptosis and elevated soluble FAS ligand were more characteristic to patients with *FAS* gene mutation.

In conclusion, we evaluated a large cohort of orphan disease ALPS patients describing the most frequent clinical and laboratory findings. We evaluated the sensitivity and specificity of different biomarker combinations in context to the identified genetic variants.

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8. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

8.1 Publications that provide the basis of this thesis

Molnár E, Radwan N, Kovács G, Andrikovics H, Henriquez F, Zarafov A, Hayman M, Linzner D, Thrasher AJ, Buckland M, Burns SO, Gilmour KC. Key diagnostic markers for autoimmune lymphoproliferative syndrome with molecular genetic diagnosis. Blood. 2020 Oct 22;136(17):1933-1945. doi: 10.1182/blood.2020005486. PMID: 32599613. [Impact factor: 17.543]

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8.2 Other publications by the candidate

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