A survey of 90 patients with autoimmune lymphoproliferative syndrome related to
TNFRSF6 mutation

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Running title: ALPS related to TNFRSF6 mutation

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Abstract:
Autoimmune lymphoproliferative syndrome (ALPS) is a genetic disorder characterized by early-onset, chronic, non-malignant lymphoproliferation, autoimmune manifestations and susceptibility to lymphoma. The majority of ALPS patients carry heterozygous germline (ALPS-FAS) or somatic mutations (ALPS-sFAS) of the TNFRSF6 gene coding for FAS. Although the clinical features of ALPS have been described, long-term follow-up data on morbidity and mortality are scarce. We performed a retrospective analysis of clinical and genetic features of 90 ALPS-FAS and ALPS-sFAS patients monitored over a median period of 20.5 years. Heterozygous germline mutations of TNFRSF6 were identified in 83% of probands. Somatic TNFRSF6 mutations were found in 17% of index cases (all located within the intracellular domain of FAS). Sixty percent of the ALPS-FAS patients with mutation in the extracellular domain had a somatic mutation affecting the second allele of TNFRSF6; age at onset was later in these patients. No other genotype-phenotype correlations could be found. Long-term analysis confirmed a trend towards spontaneous remission of lymphoproliferation in adulthood but mixed outcomes for autoimmune manifestations. We observed significant and potentially life-threatening disease and treatment-related morbidity including a high risk of sepsis after splenectomy that invites to careful long-term monitoring of ALPS patients. We also noted a significantly greater occurrence of disease-related symptoms in males than in females.
Autoimmune lymphoproliferative syndrome (ALPS, also known as Canale-Smith syndrome) is a genetic disorder characterized by early-onset, chronic, non-malignant lymphoproliferation, autoimmune manifestations and susceptibility to lymphoma. In ALPS, lymphocyte homeostasis is disrupted by defects in the apoptosis mediated by Fas, a cell-surface receptor (also referred to as Apo-1 and CD95) from the tumour necrosis factor receptor superfamily. High counts of circulating TCRαβ+, CD4- CD8- double-negative (DN) T lymphocytes and elevated plasma levels of Fas ligand (FasL) and interleukin (IL)10 are diagnostic hallmarks of the disease. The majority of ALPS patients carry heterozygous germline mutations in the TNFRSF6 gene coding for Fas (ALPS-FAS). Interestingly, somatic TNFRSF6 mutations represent the second most common genetic aetiology of ALPS (ALPS-sFAS). In addition, germline mutations in the genes coding for FasL, caspase 10 and caspase 8 and somatic mutations in NRAS and KRAS have been identified in a small proportion of patients with ALPS and related disorders. The classification and diagnostic criteria for ALPS have recently been revised.

The majority of ALPS-FAS related mutations are located in the Fas intracellular domain (ICD) and within latter's death domain (DD) in particular. The reported clinical penetrance of ICD mutations is high (> 80%) because the mutant proteins exert dominant-negative effects on wild-type CD95 protein. In contrast, mutations located in the Fas extracellular domain (ECD) usually display lower clinical penetrance (20-30%) due to haploinsufficiency. These observations suggest that a second event is necessary for disease expression in such cases. In fact, we recently reported on seven patients with both a germline ECD mutation and a somatic event affecting the second TNFRSF6 allele (a somatic mutation or duplication of the mutant allele with loss of the wild-type allele). The clinical phenotype of affected relatives is also reportedly milder in families with ECD mutations. Although the clinical features of ALPS have been described, data on long-term morbidity and mortality are scarce. Here, we report on the clinical and genetic features of 79 ALPS-FAS and 11 ALPS-sFAS patients monitored over a median follow-up period of 20.5 years (range: 1.5 to 77).

Patients and methods

Inclusion criteria

All index cases were referred to the Primary Immunodeficiency Study Centre at Necker Children's Hospital (Paris, France) because of suspected ALPS (defined as chronic, non-malignant lymphadenopathy and/or splenomegaly with an elevated DN T cell count (CD3+ TCRαβ+ CD4- CD8- >= 2.5%) in the peripheral blood. Patients with defective in vitro Fas-
induced lymphocyte apoptosis and/or elevated plasma FasL were screened for germline
TNFRSF6 mutations using genomic DNA. If no mutations were found, genomic DNA
extracted from sorted DN T cells was sequenced as a screen for mosaic mutations. Only
patients with confirmed, heterozygous, germline mutations (ALPS-FAS) and somatic
TNFRSF6 mutations (ALPS-sFAS) were included in this study. All participants or their
parents/guardians gave their signed, informed consent to participation in accordance with the
Declaration of Helsinki. Patients were subsequently cared for in 18 medical centres across
France and Belgium. Clinical data were collected from medical charts (reviewed by two
physicians: BN and BF) and in physician interviews.

Relatives of index cases underwent genetic and immunological screening. Laboratory tests
(such as detection of DN T cells and plasma FasL and in vitro lymphocyte T cells apoptosis
assays) were performed whenever possible. Phenotypic features (splenomegaly, adenopathy
and overt autoimmunity) were described by reviewing medical records (when available)
and/or performing physician interviews. On the basis of these informations, relatives were
classified as asymptomatic carriers or symptomatic relatives. Three first-degree relatives with
ALPS-related symptoms (chronic splenomegaly and/or lymphadenopathy with autoimmunity
and/or lymphoma in one occasion) were included in the study despite the absence of data on
TNFRSF6 mutations. A further two relatives were excluded from the study, due to the lack of
medical information. The study end date for inclusion and data collection was September
2010.

**Diagnostic procedures**

The percentage of DN T cells, plasma IL-10 and FasL levels and lymphocyte T cells
apoptosis were assessed as described previously. For the mutation analysis, DNA was
isolated by proteinase K digestion and phenol-chloroform extraction. Genomic DNA
segments were amplified as described previously. In families with incomplete clinical
penetrance of germline TNFRSF6 mutations or in patients with de novo germinal mutations in
the Fas ECD, additional somatic TNFRSF6 mutations were screened for after DN T cell
sorting, as reported recently.

**Statistics**

All statistical analyses were performed using Prism software (GraphPad Software Inc., San
Diego, CA, USA). Groups were compared in a Mann-Whitney test.
Results

Identification of TNFRSF6 mutations in index cases (Fig. 1a,b,c)
A total of 63 index cases were analyzed. Fifty-two probands (82%) carried a heterozygous germline TNFRSF6 mutation. There were 12 probands with ECD mutations (23%), one with a transmembrane domain (TMD) mutation and 39 with ICD mutations (75%), as shown in Figure 1a and b. Ten of these ECD mutations had not been reported elsewhere and are thus highlighted in Fig. 1a. Six of the ICD mutations were found in at least 2 unrelated index cases. In all, there were 26 different ICD mutations, 14 of which had not been reported elsewhere (Fig. 1b). In addition, 11 patients had mosaic ALPS (18%) and displayed somatic TNFRSF6 mutations (all in the ICD) in DN T cells. Somatic changes in the second TNFRSF6 allele were screened for in 10 of the 13 ALPS index cases carrying ECD/TMD mutations and were identified in six of these (60%). As previously reported, a somatic mutation affecting the second TNFRSF6 allele was found in two patients. Duplication of the mutant allele with loss of the wild-type allele was identified in four cases (two of which had been reported). Six of the 39 index cases with germline ICD mutations and low clinical penetrance were screened for somatic changes of the second TNFRSF6 allele. Loss of the wild-type allele was identified in one of these cases.

Family analysis
Genetic analysis of family members was performed for 46 of the 52 patients carrying germline TNFRSF6 mutations. No clinical or immunological features of ALPS were detected in the parents of the 9 patients with de novo germline mutations. Inherited mutations were found in 37 families, with maternal and paternal inheritance in 19 and 18 cases, respectively. The mutation was also detected in 5 grandparents, 17 siblings, 3 children and 9 other relatives. Of the 71 individuals with germline TNFRSF6 mutations, 24 had a positive medical history and biological markers of ALPS, whereas 40 adults and 7 children were asymptomatic. Two relatives (1 grandfather and 1 sibling) also had a typical medical history of ALPS but were not available for genetic testing. Six families were not investigated. In one of the latter families, medical history was suggestive of ALPS; the proband’s mother had presented Hodgkin’s lymphoma (HL) in childhood and benign lymphoproliferation in adulthood. In all, 27 symptomatic relatives were identified and included in the clinical survey.
Seven symptomatic relatives carried ECD/TMD mutations. Somatic changes in the second $TNFRSF6$ allele were screened for in three of these subjects and confirmed in two. Both cases corresponded to duplication of the mutant allele with loss of the wild-type allele.

**Clinical features**

The demographic and clinical characteristics of the population are summarized in Table 1. At last follow-up, the median age of all patients (i.e. index cases and symptomatic relatives) was 22.5 years (range: 1.9-77 years). The male/female gender ratio was 2.2. Index cases ($n= 63$) had a median age of 17.6 years (1.9-48 years) with a gender ratio of 2.1. Symptomatic relatives ($n= 27$) had a median age of 37.5 years (1.2-77 years) and gender ratio of 2. Asymptomatic family members had a median age of 43.7 years (5-73 years) and a gender ratio of 0.7. Higher penetrance in males was suggested by the fact that 75% of the genetically affected males developed ALPS, compared with 50.8% of females ($p< 0.01$).

The median age at symptom onset was 3 years (0 to 35) (Fig. 2a). However, 7 of the 90 patients (7%) had late onset disease (i.e. between the ages of 18 and 35 years.) Patients with late-onset disease displayed milder lymphoproliferation but showed active autoimmune manifestations. The first disease manifestations are depicted in Fig. 2b.

**Lymphoproliferation**

Chronic lymphoproliferation was present in 89 of the 90 subjects. Splenomegaly was the predominant feature (85 cases, 94%) and generally occurred early in the course of the disease (Fig. 3a). Hypersplenism was noted in 70% of the patients (58 out of 80). Splenic fracture occurred in one individual. One patient presented hydrops fetalis at birth as a consequence of massive prenatal lymphoproliferation and cytopenia. Chronic lymphadenopathy was also frequent (in 72 out of 85 patients) but developed later than splenomegaly did (Fig. 3b). Two patients presented with dysphonia related to histologically confirmed laryngeal lymphoproliferation.

**Autoimmunity (Table 2 and Fig. 3)**

Autoimmune manifestations occurred in 55 of the 90 patients (61%) at a median age of 6.25 years (0-30 years). The risk of developing autoimmunity before the age of 30 was calculated to be 72% (Fig. 3c). A detailed analysis of the autoimmune manifestations is provided in Table 2. Autoimmune cytopenia was the main manifestation and occurred in 47 of the 90 patients (52%) at a median age of 5 (0.25-30) years. Autoimmune haemolytic anaemia was
the most frequent event (n= 30). Twenty-three patients had autoimmune thrombocytopenia (platelet count < 30,000/μl). Profound autoimmune neutropenia was diagnosed in 7 cases (<200/μl). Sixteen other (later-onset) autoimmune manifestations occurred in 12 patients (14.4%) at a median age of 12.5 years (0.25-24 years). Five of the latter events were preceded by or occurred concomitantly with autoimmune cytopenia. Two patients developed glomerulonephritis, with spontaneous recovery from transient acute kidney failure in one case and nephrotic syndrome requiring therapy in the other. Kidney biopsies from both patients revealed mesangiopathic glomerulonephritis with crescent formation and minor IgA deposits. Three patients presented acute seronegative hepatitis, which resolved either spontaneously (n=2) or after a short course of steroids (n=1). Aplastic anaemia occurred in 2 patients, with nodular lymphoid infiltration (mostly by DN T cells). Chronic pancreatitis occurred in one patient. Another patient presented severe osteopenia at the age of 6 years (osteodensitometry of the femur and spine: -7 SD), which led to pain, multiple bone fractures and growth failure. This patient has never been treated with steroids. Osteopenia dramatically improved after immunosuppressive therapy that included cyclophosphamide, vincristin, steroids and alemtuzumab. Hematopoietic stem cell transplantation (HSCT) was attempted but the graft was rejected. Significant improvement of osteopenia after immunusuppression suggested autoimmune origin. Concomitant pulmonary and skin vasculitis occurred in 1 patient. Partial, transient alopecia was noted once. One patient presented recurrent angio-oedema and urticaria. Lastly, recurrent episodes of rash (reported as polymorphic maculopapular rash or giant urticaria) were reported in 20% of the patients.

Laboratory results
Data on IgG, IgA and IgM serum levels were available for 73 patients. Almost all patients presented hyper IgG (71 out of 73) and hyper IgA serum levels, although the phenotype varied markedly from one patient to another and over time for a given individual (data not shown). Two patients presented a progressive hypo IgG at the age of 15 years, both of whom required immunoglobulin substitution, none of these 2 patients received previous immunosuppressive drugs despite active lymphoproliferation from infancy. IgM values were within the normal range in 35 of the 73 patients and below the normal range in 38. Hypo IgM serum level was present at diagnosis in half of the cases and appeared over time in the other cases.

Disease management
Sixty-four of 86 patients (74%) required medical and/or surgical treatment at some point during the follow-up period. Thirty patients (33%) underwent complete splenectomy because of massive splenomegaly (n= 12) and/or refractory cytopenia/autoimmunity (n= 18). Nineteen of the 63 index cases (30%) were splenectomized at a median age of 6.5 years (0.5-17 years) and 11 of the 27 symptomatic relatives (41%) at a median age of 15 years (2-43 years). Splenectomy was initially efficient to treat cytopenia but relapse of autoimmunity occurred in half of the patients. Fifty-two patients (52/86= 60%) were treated with immunosuppressive drugs. Immunosuppression was primarily initiated because of autoimmunity in 80% and for lymphoproliferation (abdominal pain related to voluminous splenomegaly and/or massive lymphadenopathies) in the remaining 20%. Median age at initiation of medical treatment was 7.5 years (mean 9.1, range 0.1-29) for a median duration of 3 years (mean 5.5, range 0.5-23). The various immunosuppressive treatments administered were based mainly on steroids (n= 42), 6-mercaptopurine (6MP) (n= 25), azathioprine (n= 19), mycophenolate mofetil (n= 5), rapamycin (n= 3), and anti-CD20 monoclonal antibodies (n= 9). Pulse of cyclophosphamide (n= 3) and plasmapheresis (n= 2) were rarely attempted in severe and refractory autoimmunity (AIHA, ITP, severe osteopenia, hyperviscous syndrome related to hypergammaglobulinemia). HSCT was attempted in 1 patient for severe osteopenia (see above) but the graft was rejected. Efficacy of immunosuppressive drugs cannot be analysed in deep in such a retrospective analysis where several therapeutic modalities were used. It can nevertheless be restated that steroids were the front-line therapy for autoimmune manifestations. Second lines were mainly 6MP, azathioprine or MMF. Additional lines of therapy included anti-CD20 monoclonal antibody, rapamycin or combination of therapies. In the past, Splenectomy was initially proposed as second or third line of treatment. In recent years, this procedure was avoided because of the increased risk of invasive infection with encapsulated bacteria in this setting of patients (described below). Among 45 patients treated for autoimmunity, 7 received one line of therapy, 13 patients had 2 and 25 patients ≥ 3 lines of treatments. Anti-CD20 monoclonal antibodies were used in 9 patients with autoimmune cytopenia. As previously reported24, efficacy in treatment of AIHA was disappointing (no effect n= 3 or partial improvement n= 3). Three patients with ITP achieved remission after rituximab, but 2 relapsed few months later. Lymphoproliferation and hypersplenism were efficiently treated with 6MP, azathioprin or in few occasions, sirolimus.

Malignancy
A total of 7 lymphomas occurred in 3 index cases and 3 symptomatic relatives. Median age at diagnosis was 24.5 years (range 14-51). There were 3 cases of Hodgkin’s lymphoma (HL) and 4 cases of B cell non-Hodgkin’s lymphoma (NHL). One case of B-cell NHL has been reported previously. One patient presented concomitant large B cell NHL in the duodenum and HL in the bone marrow. One B cell NHL case was EBV positive. All but one of the six patients had been treated for ALPS-related symptoms from early childhood onwards. One patient had presented mild undiagnosed lymphoproliferation before developing a lymphoid malignancy. All cases were treated with conventional chemotherapy, except for the case of EBV-related lymphoma in which immunotherapy only was administered (anti-CD20 monoclonal antibodies and reduction of the immunosuppression). All patients with follow up >5 years (n=4) showed long-term remission. The cumulative risk of developing lymphoid malignancy before the age of 30 was calculated to be 15% (Fig. 4b). Low-grade glioma occurred in one patient at the age of 19. It was successfully treated by radiotherapy, with a follow-up of 5 years.

**Long-term disease progression (Table 3)**

We analyzed the long-term data for a sub-group of informative patients who were symptomatic before the age of 15 and followed up beyond the age of 20 years. Twenty-six index cases and 16 symptomatic relatives met these criteria (n= 42). The data are shown in Table 3. The median duration of follow-up for these 42 patients was 28 years (mean: 32; range 20.5-77 years). All 42 subjects presented lymphoproliferation (100%) and 25 (59%) developed autoimmune phenomena during childhood. Twenty received drug treatment during childhood (48%). Lymphoproliferation improved in all subjects, with complete remission in 28 cases (66%) and significant improvement in the others (34%). Remission of autoimmunity was observed in 44% of cases. Autoimmune cytopenia remained active in 14/25 patients (56%). Despite the fact that autoimmune manifestations were present in a milder form in half of them, all required continuation of immunosuppressive drugs in adulthood. Overall, these data confirmed a trend towards the progressive remission of lymphoproliferation in adulthood. However, autoimmune manifestations remained active in a setting a patients and imposed long lasting immunosuppressive drugs in adulthood.

**Disease activity and treatment-related events (Fig. 3)**

Nine of the 30 splenectomized patients (30%) developed 17 severe, invasive bacterial infections. Only one patient was receiving immunosuppressive treatment when infection
occurred. Four patients died as a consequence of these infections, giving a mortality rate for invasive bacterial infection after splenectomy of 13.3%. The infections occurred 1.8 to 44 years following splenectomy (median: 10 years). The main pathogen was *Streptococcus pneumoniae*. *Streptococcus agalactiae* was identified in one adult male patient. No microorganisms were identified in 3 cases. Age at splenectomy appeared to be significant risk factors for invasive bacterial infections: 4 out of 6 patients splenectomised ≤ 5 years of age developed 1 to 5 invasive bacterial infections while 5 of 24 patients splenectomised ≥ 5 years developed one episode of invasive bacterial infections (p <0.05). There was also a trend for increased risk of invasive bacterial infection in patients with poor adherence to usual prophylactic recommendations (antibioprohylaxis during minimum 5 years following splenectomy in children, 2 years in adults and all life updated vaccination against St pneumoniae, H influenzae and N. Meningitidis26): 3 of 6 non compliant patients versus 6/21 adherent patients developed invasive bacterial infection. Immunosuppressive treatment prior to or at the time of infection occurrence did not appear as a risk factor. Only one patient was receiving 6MP at onset of infection. Five of 16 patients who received immunosuppression prior to or following splenectomy developed an invasive bacterial infection versus 4/14 who did not received any immunosuppression. Considering splenectomised patients without any risk factors (> 5 years at splenectomy and good compliance to prophylactic recommendations), 3/18 (17%) patients developed infections. Subcutaneous abscesses were observed in one patient suffering from autoimmune neutropenia. *Pneumocystis jiroveci* pneumonia occurred in two patients on immunosuppressants (steroids, 6 MP, azathioprine and anti CD20 monoclonal antibody in one, steroids, 6MP and anti CD20 monoclonal antibody in the other). Epidermodysplasia verruciformis (related to chronic human papillomavirus type 5 infection) occurred in one patient 4 years after an unsuccessful HSCT, while she was being treated with 6MP. The patients did not suffer from any other recurrent or severe infectious complications.

Growth failure was observed in 21 index cases (33%). In three cases, growth failure occurred early (before the age of 5) and concomitantly with severe autoimmunity. This condition was treated with long-term steroid therapy. One patient had a growth arrest at the age of 6 years as a consequence of severe (and presumably autoimmune) osteopenia (see above). Among 17 patients with short stature and delayed puberty at teenage, 5 received long term low dose steroids from early childhood (< 0.3 mg/kg/day) that was on going at teenage, 5 never received any steroids and 7 received short course steroids or steroids > 3 months but in early childhood (< 8 years old). None received pulse steroids. When considering index cases over
the age of 20 years at last follow-up (n= 26), 42% (11/26) had growth retardation during adolescence. All presented catch-up growth in parallel to development of puberty with adult height in the normal range.

Six patients (3 index cases and 3 symptomatic relatives) died during the study period at a median age of 25 years (1.5-40). The main cause of death was post-splenectomy infection (n=4). One patient with aplastic anaemia died at the age of 18 months. The last patient died of a stroke at the age of 35 years (Fig. 4b).

Analysis of genotype/phenotype correlations and penetrance
We analysed the cohort's clinical parameters as a function of genotype (germline ECD and TMD mutations (n=20), germline ICD (n= 59) and mosaic ICD mutants (n=11)), as shown in Table 4. ALPS-FAS patients with associated somatic alterations of the second TNFRSF6 allele were also analysed separately. Age at disease onset as a function of genotype is shown in Fig. 5. Patients with combined germline and somatic TNFRSF6 mutations tended to be older at disease onset than other ALPS-FAS patients (p= 0.045) or ALPS-sFAS (p= 0.01) patients. Similarly, patients with germline ECD mutations tended to be older than patients with ICD mutations and ALPS-sFAS at the time of occurrence of the first disease-related symptoms. The groups did not differ in terms of the presence of autoimmune manifestations, as shown in Table 4. Lymphoma occurred in patients from each of the genetic groups. Likewise, the groups did not differ in terms of clinical parameters (lymphoproliferation and autoimmunity) and disease severity (significant morbidity, need for treatment and remission).

Interestingly, there was a male predominance among symptomatic individuals, regardless of the genotype. A male/female gender ratio of 2.9 was observed for patients with autoimmune manifestations. In patients with autoimmune cytopenia, this male predominance was even stronger (4.2 (38/9)). The gender ratio in patients presenting other autoimmune manifestations was 1.4 (7/5). Penetrance among relatives and overall penetrance (for all mutation-positive index cases and relatives) are shown in Table 4. Missense mutations of exon 9 (encoding the DD) showed the highest penetrance. It is noteworthy that wide phenotype variability was observed for ALPS patients from the same family.
Discussion

We have reported the long-term phenotypic and genotypic characteristics of the largest cohort to date of heterozygous ALPS-FAS and ALPS-sFAS patients. Unsurprisingly, lymphoproliferation and autoimmunity were the hallmarks of the disease. Our long-term follow-up study confirmed a trend towards spontaneous remission of lymphoproliferation in adulthood. This trend was not clearly observed for autoimmune manifestations that persisted in a significant fraction of patients and required long lasting immunosuppression at adulthood. We observed significant, life-threatening disease- and treatment-related morbidity. Strikingly, 17% of the index cases carried somatic \textit{TNFRSF6} mutations (all of which were located in the ICD). In accordance with previous reports, 25% of the heterozygous ALPS-FAS patients had mutations in the ECD and 75% had mutations in the ICD (with 29 out of 40 in the DD)\textsuperscript{19}. No genotype-phenotype correlations could be found, other than later age at onset in patients with combined germline \textit{TNFRSF6} mutation and a somatic genetic event affecting the second \textit{TNFRSF6} allele. We also noted a significantly greater increase in disease-related symptoms in males than in females. Overall, these findings merit additional comments.

Lymphoproliferation was the most common sign of disease onset and generally occurred early in life. However, 7 of the 90 patients (8%) presented late-onset lymphoproliferation during adulthood. Few cases with late onset have been reported in the literature\textsuperscript{27}. Although these observations are rare, they emphasize the need to consider a diagnosis of ALPS in adulthood. Autoimmune manifestations were also frequent and usually occurred a few years after lymphoproliferation. Cytopenia was the most frequently observed sign, as previously reported. The pattern of autoimmunity in ALPS patients differs from that seen in \textit{lpr} mice\textsuperscript{28-30}. Depending on the genetic background, this animal model mainly develops lupus-like autoimmune disease and nephritis. In ALPS patients, cytopenia is far more common. Glomerulonephritis occurred in only 3% of our cohort and no cases of systemic lupus erythematosus were observed.

In all patients monitored over a long period of time, lymphoproliferation decreased or disappeared at adulthood. This observation contrasts with the situation in \textit{lpr} mice, in which DN T cells accumulate over-time and the life-span is shortened\textsuperscript{28}. Active thymopoiesis and antigenic stimulation in early childhood may increase T lymphocytes load in the periphery (particularly for the CD8+ thymocytes that give rise to DN T cells)\textsuperscript{31}. Another possible explanation is the progressive involvement of an alternative, compensatory Fas-independent apoptotic mechanism. In contrast, more than half of the adult patients continued to present autoimmune complications in general and autoimmune cytopenia in particular. It may be that
self-reactive B cell clones give rise to long-lasting plasma cells capable of producing autoantibodies for a very long period of time. The contrast between the remission of lymphoproliferation and the persistence of autoimmunity suggests that lymphoproliferation is not simply the consequence of the accumulation of self-reactive clones.

Within the patient group, we observed a strikingly high incidence of severe, post-splenectomy infections (mainly due to *Streptococcus pneumoniae*) but no other susceptibility to infections. The increased risk of overwhelming, post-splenectomy infections has already been noted by Canale and Smith and others but our report is the first to have assessed this factor, the main cause of death in our cohort. Thirty percent of the splenectomised patients had at least one episode of severe infection (more than 20 years after splenectomy, in some individuals). Although this risk has been well documented in asplenic patients and splenectomised individuals, the incidence observed here is much higher than expected. Despite identified risk factors (young age at splenectomy and poor adherence to prophylactic measures), incidence of severe, post-splenectomy infections is high. The reason for this increased susceptibility is unknown and is not clearly related to addition of immunosuppressive treatment. In any event, splenectomy should be avoided in ALPS patients unless absolutely essential.

In our experience, 64 patients (86%) with ALPS required treatment. Autoimmune manifestations, in particular autoimmune cytopenia imposed immunosuppressive treatment in most cases. It’s unclear whether ALPS patients with autoimmunity should be treated in a similar manner than patients with the same autoimmune manifestation in the absence of ALPS. Use of immunosuppressive drugs with pro-apoptotic and/or anti-proliferative properties (azathioprine, MMF, 6MP, rapamycin) could be preferentially used based on disease pathophysiology. Toxicity has to be considered given the fairly high risk of recurrence. Anti CD20 antibody therapy does not appear at least for AIHA, as effective as in patients without ALPS.

It is already known that ALPS is accompanied by an increased risk of lymphoma. Thirteen cases of various lymphoid malignancies (mainly B-cell NHL and HL, including nodular lymphocyte predominant HL) were reported in 12 patients. The relative risks of developing HL and NHL were estimated to be 51 and 14. In literature reports, all affected patients carried *TNFRSF6* mutations within the ICD and particularly within the DD. In our cohort, the cumulative risk of developing lymphoid malignancy before the age of 30 years was calculated to be 15%. Importantly, some affected patients carried ECD and mosaic mutations rather than germline *TNFRSF6* ICD mutations, extending the spectrum of "at risk"
patients. Glioma has been reported previously in one ALPS patient\textsuperscript{18}. Hence, this association of two rare conditions may not have occurred by chance.

In the present study, the penetrance of ECD and ICD mutants in relatives was 28\% and 40\%, respectively, with a higher penetrance (56\%) for missense mutations inside the DD; for carriers as a whole, the penetrance values were 52\%, 63 and 73\% for ECD mutations, ICD mutations and DD missense mutations, respectively. The observed penetrance rate for ECD mutations agrees with published results\textsuperscript{18,22,35}. It is noteworthy that the penetrance of DD mutants observed here is much lower than the value of 85 to 90\% previously reported in relatives with DD missense mutations\textsuperscript{18,22,35}. We cannot strictly rule out the possibility that our retrospective analysis resulted in the underestimation of disease-related symptoms in relatives. In contrast, half of the mutations reported here are new. Although a potential dominant negative effect has not been studied for the latter, one can speculate that several lead to haploinsufficiency that is associated with lower penetrance.

The variable clinical penetrance of \textit{TNFRSF6} mutations and the differing phenotype found in patients from the same family indicate that additional genetic or environmental factors determine ALPS. Indeed, we recently reported the additional presence of a somatic event impairing the second \textit{TNFRSF6} allele (i.e. somatic mutation or duplication of the mutant allele with loss of the wild-type allele) in seven patients with germline ECD mutation\textsuperscript{19}. Five of these patients were included in this survey and four additional patients were identified. Co-mutation was frequently found in ALPS-FAS patients carrying ECD mutations (8 out of 12, 66\%). The latter are known to induce loss of Fas expression at the cell membrane and therefore produce haploinsufficiency\textsuperscript{19}, the second mutation leading to loss of membrane FAS expression. Similar events were screened for in selected patients carrying ICD mutants with low clinical penetrance but were rarely identified (n=1 out of 6). This latter patient carried a germline frameshift mutation at the end of exon 9 with somatic telomeric uniparental disomy and displayed a complete Fas expression defect in DN T cells. Interestingly, germline mutation patients with somatic changes in the second \textit{TNFRSF6} allele were significantly older at disease onset than other ALPS-FAS or ALPS-sFAS patients were; this suggests that the second "hit" may be acquired later in life. Overall, the absence of genotype-phenotype correlation suggests that other factors dictate the occurrence of autoimmunity and the disease severity. Phenotypic heterogeneity is also observed in mice carrying \textit{TNFRSF6} mutation, with the autoimmune manifestations varying as a function of the genetic background \textsuperscript{28}.

We observed significant male predominance among index cases and symptomatic relatives and female predominance in asymptomatic relatives. These gender ratios did not appear to
depend on the genotype characteristics, although the number of ALPS-sFAS patients was small. Interestingly, Dowdell et al.\textsuperscript{36} reported a series of 12 ALPS-sFAS with a male/female gender ratio of 3. Bleesing et al.\textsuperscript{37} found a male/female gender ratio of 1.8 in ALPS-FAS probands. This observation contrasts with the female predominance found in many autoimmune diseases\textsuperscript{38} and suggests the involvement of a predisposing genetic factor on the Y chromosome or a recessive X-linked factor.

This study of a large cohort of ALPS-FAS and ALPS-sFAS patients highlighted the significant morbidity of this disease, which was mainly related to long-term autoimmune manifestations and an increased risk of lymphoma. It also emphasized the high risk of post-splenectomy invasive bacterial infections and related mortality, which might suggest the existence of specific susceptibility in this patient population. Further prospective studies are needed to determine the risk factors for developing ALPS symptoms and thus appropriate treatments for individual patients.

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\textbf{Conflict of interest :} None

\textbf{Authors contributions:} B.N. designed the research, collected and analyzed data, participated to writing of the paper and to clinical care of the patients; A.M.C. performed genetic and biologic diagnosis of the patients, analyzed data and critically read the manuscript; B.F., D.G., O.L. and L.D.S. collected and analyzed data, participated to clinical care of the patients and
critically read the manuscript; N.L. and M.C.S. participated to biologic diagnosis of the patients; B.B.M, N.A., C.C, Y.B., E.J., G.L., G.M., F.S., E.O., O.H., S.B. participated to clinical care of the patients; C.P. participated to genetic and biologic diagnosis of the patients and to patient’s care, A.F. participated to analyses of data, writing of the paper and clinical care of the patients; F.R.L. participated to genetic and biologic diagnosis of the patients, analyses of data and writing of the paper.
References


Table 1: Characteristics of the population and clinical features

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>Index cases</th>
<th>Symptomatic relatives</th>
<th>Asymptomatic relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N= 90</td>
<td>n=63</td>
<td>n=27</td>
<td>n= 47</td>
</tr>
<tr>
<td>Median age (range, years)</td>
<td>22.5 (1.9-77)</td>
<td>17.6 (1.9-48)</td>
<td>37.5 (1.2-77)</td>
<td>43.7 (5-73)</td>
</tr>
<tr>
<td>Sex ratio (male/female)</td>
<td>62/28 (2.2)</td>
<td>44/19 (2.1)</td>
<td>18/9 (2)</td>
<td>20/27 (0.7)</td>
</tr>
<tr>
<td><strong>Lymphoproliferation (LP)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign chronic* LP</td>
<td>89/90 (99)</td>
<td>62/63 (98)</td>
<td>27/27 (100)</td>
<td>0</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>85/90 (95)</td>
<td>59/63 (93)</td>
<td>26/27 (95)</td>
<td></td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>76/84 (90)</td>
<td>58/63 (92)</td>
<td>18/21 (85)</td>
<td></td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>ND</td>
<td>33/63 (50)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td><strong>Autoimmunity (AI)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any AI symptoms (%)</td>
<td>55/88 (63)</td>
<td>41/63 (65)</td>
<td>14/25 (56)</td>
<td>0</td>
</tr>
<tr>
<td>AI cytopenias (%)</td>
<td>47/88 (53)</td>
<td>34/63 (55)</td>
<td>13/25</td>
<td></td>
</tr>
<tr>
<td>Other AI symptoms (%)</td>
<td>13/88 (15)</td>
<td>12/63 (20)</td>
<td>1/25</td>
<td></td>
</tr>
<tr>
<td><strong>Neoplasia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>8/90 (9)</td>
<td>5/63 (8)</td>
<td>3/27 (11)</td>
<td>0</td>
</tr>
<tr>
<td>B cell NHL</td>
<td>4/90</td>
<td>3/63</td>
<td>1/27</td>
<td></td>
</tr>
<tr>
<td>HL</td>
<td>3/90</td>
<td>1/63</td>
<td>2/27</td>
<td></td>
</tr>
<tr>
<td>Glioma</td>
<td>1/90</td>
<td>1/63</td>
<td>0/27</td>
<td></td>
</tr>
</tbody>
</table>

*: > 6 months. LP denotes lymphoproliferation; AI: autoimmune or autoimmunity; NHL: non Hodgkin’s lymphoma, HL: Hodgkin’s lymphoma; ND: not determined
Table 2: Autoimmune manifestations in ALPS-FAS and sFAS patients (n= 90)

<table>
<thead>
<tr>
<th>Any AI manifestations (n= 77 in 55 patients)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 AI manifestation</td>
<td>37</td>
</tr>
<tr>
<td>2 AI manifestations</td>
<td>14</td>
</tr>
<tr>
<td>3 AI manifestations</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AI cytopenias (n= 60 in 47 patients)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AIHA</td>
<td>30</td>
</tr>
<tr>
<td>ITP</td>
<td>23</td>
</tr>
<tr>
<td>AIN</td>
<td>7</td>
</tr>
<tr>
<td>One cytopenia</td>
<td>36</td>
</tr>
<tr>
<td>Bicytopenia</td>
<td>9</td>
</tr>
<tr>
<td>Tricytopenia</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other AI manifestations (n= 16 in 12 patients)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerulonephritis</td>
<td>2</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>3</td>
</tr>
<tr>
<td>Vasculitis</td>
<td>4</td>
</tr>
<tr>
<td>Uveitis</td>
<td>1</td>
</tr>
<tr>
<td>Aplastic anemia</td>
<td>2</td>
</tr>
<tr>
<td>Chronic pancreatitis</td>
<td>1</td>
</tr>
<tr>
<td>Severe osteopenia</td>
<td>1</td>
</tr>
<tr>
<td>Angioedema</td>
<td>1</td>
</tr>
<tr>
<td>Transient alopecia</td>
<td>1</td>
</tr>
</tbody>
</table>

AI: autoimmune; AIHA: autoimmune haemolytic anemia; ITP: immune thrombocytopenic purpura; AIN: autoimmune neutropenia
<table>
<thead>
<tr>
<th></th>
<th>&lt; 15 years</th>
<th>&gt; 20 years</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at onset (median-range)(yrs):</strong></td>
<td>3 (0-14)</td>
<td>28 (20.5-77)</td>
</tr>
<tr>
<td><strong>Lymphoproliferation</strong></td>
<td>42 (100)</td>
<td>28 (66)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 (34)</td>
</tr>
<tr>
<td><strong>Autoimmunity</strong></td>
<td>25 (59)</td>
<td>11 (44)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 (56)</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td>26 (48)</td>
<td>12 (46)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 (54)</td>
</tr>
</tbody>
</table>

Yrs: years;
## Table 4: Genotype/Phenotype Correlation and Penetrance

<table>
<thead>
<tr>
<th></th>
<th>ALPS-FAS ECD/TMD mutations</th>
<th>ALPS-FAS ICD mutations</th>
<th>ALPS-sFAS</th>
<th>Combined ALPS-FAS/sFAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>N= (index cases/sympt. Relatives)</td>
<td>20 (13/7)</td>
<td>59 (39/20)</td>
<td>11 (11/0)</td>
<td>8 (6/2)</td>
</tr>
<tr>
<td>Age (yrs) at last follow up (median-range)</td>
<td>20.6 (1-47)</td>
<td>24 (1.5-77)</td>
<td>17 (3-30)</td>
<td>20.9 (12-47)</td>
</tr>
<tr>
<td>Age (yrs) at onset (median-range)</td>
<td>6 (0.2-29)</td>
<td>3 (0-30)</td>
<td>2 (0.1-5)</td>
<td>11 (0.2-27)</td>
</tr>
<tr>
<td>Sex ratio in sympt. Individuals (M/F)</td>
<td>2 (14/6)</td>
<td>2 (39/20)</td>
<td>1.8 (7/4)</td>
<td>3 (6/2)</td>
</tr>
<tr>
<td>Sex ratio in asympt. carriers (M/F)</td>
<td>0.6 (7/11)</td>
<td>0.8 (13/17)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Lymphoproliferation (%)</td>
<td>20 (100)</td>
<td>58 (98)</td>
<td>11 (100)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Autoimmunity (%)</td>
<td>12 (60)</td>
<td>35 (60)</td>
<td>5 (45)</td>
<td>5 (62.5)</td>
</tr>
<tr>
<td>Lymphoma (%)</td>
<td>1 (5)</td>
<td>4 (7)</td>
<td>1 (9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Significant ALPS related morbidity* (%)</td>
<td>12 (60)</td>
<td>34 (57)</td>
<td>7 (63)</td>
<td>6 (75)</td>
</tr>
<tr>
<td>Treatment (%)</td>
<td>12 (60)</td>
<td>40 (68)</td>
<td>8 (72)</td>
<td>7 (90)</td>
</tr>
<tr>
<td>Remission** (%)</td>
<td>8 (40)</td>
<td>20 (34)</td>
<td>3 (27)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Penetrancess in relatives (%)</td>
<td>7/25 (28)</td>
<td>20/50 (40)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Penetration in all carriers (%)</td>
<td>20/38 (52)</td>
<td>50/79 (63)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* patients undergoing splenectomy, having autoimmunity requiring treatment or developing lymphoma. **Absence of lymphoproliferation and autoimmunity without treatment at last follow up for > 1 year. ALPS-FAS and ALPS-sFAS: ALPS patients with germline and somatic heterozygous *TNFRSF6* mutation respectively.
Legend of figures

**Figure 1: Genotypic characteristics of ALPS-FAS and ALPS-sFAS cases**
The structure of the *TNFRSF6* gene showing ALPS-FAS-associated mutations in exons 1 to 6 (Fig. 1a) encoding the extracellular domain (ECD) and transmembrane domain (TMD) and exons 7 to 9 (Fig. 1b) encoding the intracellular domain (ICD). DD denotes the death domain.

Mutations are numbered with respect to the immature protein. The symbols (●,▲) denote missense and frameshift mutations respectively; deletions and nonsense mutations are also indicated. Mutations denoted by with * are novel. The numbers of unrelated index cases carrying the same mutation are given in brackets. Mutations associated with ALPS-sFAS are shown in Fig. 1c.

**Figure 2: Age at onset and the nature of the first manifestations of ALPS**
The age distribution at disease onset for 84 patients (Fig. 2a). Seven patients developed the disease after the age of 18 years.

The distribution of the first symptoms of ALPS (Fig. 2b).

**Figure 3: Cumulative frequency of patients having developed (a) splenomegaly, (b) lymphadenopathy and (c) autoimmunity, as a function of age**
Splenomegaly occurs early in childhood and precedes the development of lymphadenopathy and autoimmune manifestations. ++ denotes lymphadenopathy > 2 cm and < 5 cm. +++ denotes lymphadenopathy > 5 cm. More than 90% of patients developed splenomegaly. Significant lymphadenopathy and autoimmunity were observed in 72 and 75% of the patients, respectively.

**Figure 4: Event-free survival and lymphoma-free survival in the cohort of patients**
Event-free survival (fig 4a) and lymphoma-free survival (fig 4b) among the cohort of patients.

**Figure 5: Age at onset of ALPS as a function of genotype**
Patients with germline TNFRSF6 mutation and a somatic event impairing the second TNFRSF6 allele (“bi-allelic” patients) had a later onset than germline-only ALPS-FAS or ALPS-sFAS patients, as shown in Fig. 1a. ALPS-FAS patients with germline ECD mutations (including patients with *TNFRSF6* “bi-allelicism”) tended to be older at ALPS onset than
patients with ICD mutation were, although this difference was not statistically significant. ALPS-sFAS patients had the lowest age at ALPS onset (*= p< 0.05; ns: non-significant).
Figure 1a

- del ex3
- Q57X*
- E79X
- "del ex 6"
- C129del
- C157X*
- D66G*
- Y91C*
- C104G*
- D108G*
- E116G*
- ●●●●
- Q57X*
- D121fs X150*
- F134fs X186*
- 1836
- 314-336
Figure 2

(a) Median age: 3 years (0 and 30 years)

(b) Lymphoproliferation: n=62 (69%)
   Autoimmunity: n=3 (3.4%)
   Lymphoma: n=2 (2.6%)
   Lymphoproliferation autoimmunity: n=23 (25%)
Figure 3

(a) Percent with splenomegaly

(b) % patients with ADP (++ and +++), n = 85

(c) Percent with autoimmunity

n = 90

age (years)
Figure 4

(a) Survival (%)

(b) EFS (lymphoma)

Time (years)
A survey of 90 patients with autoimmune lymphoproliferative syndrome related to \textit{TNFRSF6} mutation

Bénédicte Neven, Aude Magerus-Chatinet, Benoit Florkin, Delphine Gobert, Olivier Lambotte, Lien De Somer, Nina Lanzarotti, Marie-Claude Stolzenberg, Brigitte Bader-Meunier, Nathalie Aladjidi, Christophe Chantrain, Yves Bertrand, Eric Jeziorski, Guy Leverger, Gérard Michel, Felipe Suarez, Eric Oksenhendler, Olivier Hermine, Stéphane Blanche, Capucine Picard, Alain Fischer and Frédéric Rieux-Laucat