Sequential decisions on *FAS* sequencing guided by biomarkers in patients with lymphoproliferation and autoimmune cytopenia

Anne Rensing-Ehl,[1]* Ales Janda,[1]* Myriam R. Lorenz,[3] Beryl P. Gladstone,[2] Ilka Fuchs,[1] Mario Abinun,[6,19] Michael Albert,[7] Karina Butler,[8] Andrew Cant,[6,19] Anna-Maria Cseh,[9] Martin Ebinger,[10] Sigune Goldacker,[1] Sophie Hambleton,[6,19] Holger Hebart,[11] Leonora Houet,[1] Karim Kentouche,[12] Ingrid Kühnle,[13] Kai Lehmberg,[14] Ester Mejstrikova,[15] Charlotte Niemeyer,[9] Milen Minkov,[16] Olaf Neth,[17] Gregor Dückers,[18] Stephan Owens,[6] Joachim Rösler,[20] Freimut H. Schilling,[21] Volker Schuster,[22] Markus G. Seidel,[23] Petr Smisek,[15] Martina Sukova,[15] Peter Svec,[24] Thomas Wiesel,[25] Benjamin Gathmann,[1] Klaus Schwarz,[1,3,5] Werner Vach,[2] Stephan Ehl,[1,4] and Carsten Speckmann[1,4]

[1]Center of Chronic Immunodeficiency (CCI), University Medical Center Freiburg and University of Freiburg, Germany; [2]Clinical Epidemiology, Institute of Medical Biometry and Medical Informatics, University Medical Center Freiburg, University of Freiburg, Germany; [3]Institute for Transfusion Medicine, University of Ulm, Ulm, Germany; [4]Center for Pediatrics and Adolescent Medicine, University Medical Center, University of Freiburg, Germany; [5]Institute for Clinical Transfusion Medicine and Immunogenetics Ulm, German Red Cross Blood Service, Ulm, Baden–Württemberg–Hessen, Germany; [6]Department of Pediatric Immunology, Great North Children's Hospital, Newcastle upon Tyne, UK; [7]Department of Pediatric Hematology/Oncology, Dr von Haunersches Kinderspital, Munich, Germany; [8]Our Lady's Hospital for Sick Children, Dublin, Republic of Ireland; [9]Pediatric Hematology and Oncology, University Medical Center Freiburg, Germany; [10]Department of Paediatric Haematology/Oncology, University Medical Center Tübingen, Germany; [11]Department of Internal Medicine, Stauferklinikum Schwaebisch-Gmuend, Mutlangen, Germany; [12]Department of Pediatrics, Jena University Hospital, Germany; [13]Department of Pediatrics, Georg August University Göttingen, Germany; [14]Department of Pediatric Hematology/Oncology, University Medical Center Hamburg, Germany; [15]Department of Pediatric Haematology and Oncology, University Medical Hospital, 2nd Medical School, Charles University, Prague, Czech Republic; [16]St. Anna Children's Hospital, Vienna, Austria; [17]Virgen del Rocío Children's Hospital, Seville, Spain; [18]Department of Pediatrics, HELIOS-Klinikum Krefeld, Krefeld, Germany; [19]Institute of Cellular Immunology, Newcastle University, Newcastle upon Tyne, UK; [20]Department of Pediatrics, University Clinic Carl Gustav Carus, Dresden, Germany; [21]Department of Pediatric Oncology and Hematology, Olgahospital, Stuttgart, Germany; [22]Hospital for Children and Adolescents, University of Leipzig, Germany; [23]Pediatric Hematology-Oncology, Medical University of Graz, Austria; [24]Department of Pediatric Hematology and Oncology, Comenius University Children's Hospital, Bratislava, Slovakia; and [25]Vestische Kinder-und Jugendklinik Datteln, University Witten/Herdecke, Germany

©Ferrata Storti Foundation 2013

ABSTRACT

Clinical and genetic heterogeneity renders confirmation or exclusion of autoimmune lymphoproliferative syndrome difficult. To re-evaluate and improve the currently suggested diagnostic approach to patients with suspected *FAS* mutation, the most frequent cause of autoimmune lymphoproliferative syndrome, we prospectively determined 11 biomarkers in 163 patients with splenomegaly or lymphadenopathy and presumed or proven autoimmune cytopenia(s). Among 98 patients sequenced for *FAS* mutations in $CD3^+TCR\alpha/\beta^+CD4^-CD8^-$ "double negative" T cells, 32 had germline and six had somatic *FAS* mutations. The best *a priori* predictor of *FAS* mutations was the combination of vitamin B12 and soluble FAS ligand (cut-offs 1255 pg/mL and 559 pg/mL, respectively), which had a positive predictive value of 92% and a negative predictive value of 97%. We used these data to develop a web-based probability calculator for *FAS* mutations using the three most discriminatory biomarkers (vitamin B12, soluble FAS ligand, interleukin-10) of the 11 tested. Since more than 60% of patients with lymphoproliferation and autoimmune cytopenia(s) in our cohort did not harbor *FAS* mutations, 15% had somatic *FAS* mutations, and the predictive value of double-negative T-cell values was rather low (positive and negative predictive values of 61% and 77%, respectively), we argue that the previously suggested diagnostic algorithm based on determination of double-negative T cells and germline *FAS* sequencing, followed by biomarker analysis, is not efficient. We propose vitamin B12 and soluble FAS ligand assessment as the initial diagnostic step with subsequent decision on *FAS* sequencing supported by a probability-calculating tool.

Introduction

Splenomegaly and/or chronic lymphadenopathy (lymphoproliferation) and autoimmune cytopenias are common clinical problems in different immunodeficiencies and constitute a diagnostic challenge.[1] A key differential diagnosis in patients with these clinical manifestations is autoimmune lymphoproliferative syndrome (ALPS),[2] which is caused by defects in the extrinsic FAS-mediated apoptosis pathway.[3,4] Due to advances in the immunological and genetic under-

©2013 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2012.081901
The online version of this article has a Supplementary Appendix.
*ARE and AJ contributed equally to this manuscript.
Manuscript received on December 20, 2012. Manuscript accepted on July 4, 2013.
Correspondence: stephan.ehl@uniklinik-freiburg.de

standing of this disease,[5-8] the diagnostic criteria and classification have recently been revised.[9] A definitive diagnosis of ALPS requires chronic lymphoproliferation and raised levels of $CD3^+TCR\alpha/\beta^+CD4^-CD8^-$ double-negative T (DNT) cells plus one primary accessory criterion, i.e. either defective FAS-mediated T-cell apoptosis *in vitro* or a somatic or germline mutation in the genes encoding FAS (ALPS-FAS and ALPS-sFAS), FAS ligand (ALPS-FASLG) or caspase 10 (ALPS-CASP10). Patients who fulfill ALPS diagnostic criteria with defective *in vitro* FAS-induced apoptosis, in whom no causative mutation can be identified, are classified as ALPS-U. This leaves a significant proportion of patients with lymphoproliferation and autoimmunity unclassified (ALPS-phenotype: lymphoproliferation with or without autoimmune cytopenia(s), raised DNT but normal apoptosis). The latter two groups of patients may include rare cases with ALPS related diseases including caspase 8 deficiency state and RAS-associated autoimmune leukoproliferative disease.

Recently, a number of biomarkers including soluble FAS ligand (sFASL), interleukin-10 (IL-10) and vitamin B12 were shown to be specifically altered in ALPS patients[10,11] with a high positive predictive value (PPV) for the presence of *FAS* mutations.[12] Consequently, as secondary accessory diagnostic criteria for ALPS, these markers have become part of a suggested algorithm for the diagnostic work-up of patients with suspected ALPS. This algorithm recommends *FAS* germline sequencing in all patients with lymphoproliferation and elevated DNT, while further analysis for somatic *FAS* mutations depends on the biomarker profile.[9]

Despite these important advances, a number of questions remain unresolved. In the study by Caminha *et al.* at the National Institutes of Health (NIH)[12], the utility of biomarkers was retrospectively evaluated in a large cohort of ALPS-FAS, ALPS-sFAS, ALPS-U, ALPS-phenotype patients as well as mutation-positive and -negative healthy relatives. It remains to be shown whether prospective analysis of an independent cohort can confirm the high predictive value of the biomarkers. Patients were selected based on the criteria of chronic lymphoproliferation and raised DNT and thus the selection was biased by *a priori* inclusion of raised DNT as a biomarker. It therefore remains unclear how the biomarkers perform in the initial laboratory evaluation of unselected patients with lymphoproliferation and autoimmune cytopenia(s). Moreover, a number of additional biological parameters such as memory B cells, soluble CD25 (sCD25), expression of B220 on DNT and serum lipids show characteristic alterations in ALPS, but have so far not been evaluated. If their high discriminatory value can be confirmed, this may allow biomarkers to be used for initial screening with a subsequent biomarker-based decision on *FAS* sequencing. This could result in significant cost-saving.

We, therefore, initiated a prospective study to evaluate the previously assessed biomarkers as well as additional biomarkers for their ability to predict or exclude *FAS* mutations in a cohort of patients selected on the basis of their clinical presentation with splenomegaly and/or chronic lymphadenopathy and presumed or proven autoimmune cytopenia(s). We aimed to use the best identified biomarkers to develop a simple tool to calculate the individual probability of a *FAS* mutation prior to any other selection criteria such as DNT levels.

Methods

Patients

All patients presenting to one of the collaborating immunodeficiency centers with lymphoproliferation (defined as chronic non-malignant lymphadenopathy for >6 months at more than two sites and/or splenomegaly) and presumed or proven autoimmune cytopenia(s) were eligible for this study. Autoimmune cytopenia was defined as cell counts repeatedly below normal in one or more blood cell lineages with detectable autoantibodies. A number of patients with neutropenia or thrombocytopenia did not undergo autoantibody testing, but were diagnosed with presumed autoimmune cytopenia by the referring physician. The study was approved by the ethics committee of the University of Freiburg (protocol number 40/09).

Clinical chemistry, flow cytometry and apoptosis assay

Serum and blood collected into EDTA were sent to the coordinating study center in Freiburg by overnight delivery and the following 11 parameters were determined in a single reference laboratory in all patients: vitamin B12 (Eclia: Electrochemiluminescence Immunoassay, Roche Diagnostics), sCD25 (turbidimetry), high-density lipoprotein cholesterol (HDL; homogenous enzymatic color test), apolipoprotein A1 (APO-A1; turbidimetry), IgG (turbidimetry). sFASL and IL-10 were determined by an enzyme-linked immunosorbent assay as described elsewhere.[13] $CD3^+TCR\alpha/\beta^+CD4^-CD8^-$ DNT cells, the percentage of DNT cells expressing B220 (B220) and marginal zone-like ($CD19^+CD27^+IgD^+IgM^+$, MZB) and class-switched memory B cells ($CD19^+CD27^+IgD^-IgM^-$; SMB) were determined by flow cytometry as described previously.[13,14] FASL-induced apoptosis was analyzed as described elsewhere.[13]

Genetic analysis

In patients with abnormal apoptosis, exons and exon-intron boundaries of the *FAS* gene were sequenced using DNA from granulocytes or whole blood. FAS ligand (*FASLG*) and caspase 10 (*CASP10*) genes were sequenced in 60% of patients, and caspase 8 (*CASP8*) in 30%. If no mutations were found, DNA from sorted DNT cells was sequenced. In patients with normal apoptosis, sequencing was performed directly from sorted DNT cells. Somatic mutations were confirmed by sequencing of germline DNA. In some patients, we could not obtain further material for DNT sorting or could not reliably rule out somatic mutations because DNT numbers were too low. Only patients with completed genetic analysis were included in the calculation of predictive values.

Statistics

Differences in the distribution of the biomarkers across subgroups of patients were visualized using dot plots and their significance assessed by Kruskal-Wallis tests. The diagnostic values of the different markers in predicting the presence or absence of a *FAS* mutation were assessed in a first step using receiver operator characteristic (ROC) curves and corresponding area under the curve (AUC) values. Comparison of AUC values of different parameters were based on the non-parametric approach suggested by DeLong *et al.*[15] *P*-values for comparisons with published AUC values were based on inversion of confidence intervals. Biomarkers with an AUC value ≥0.7 were chosen for further evaluation. In a second step, the ability of each marker to define subgroups of clinically relevant size with positive or negative predictive values close to 100% was examined. We first considered cut-offs and combinations already suggested in the publication by Caminha *et al.*,[12] and then continued using cut-offs values chosen

©Ferrata Storti Foundation

in a way that the prevalence of positives tests corresponded to the prevalence of *FAS* mutations in our study population. These predictive values were compared with the published values using binomial tests.

All statistical analyses were done using STATA 12.[16]

Results

The majority of patients presenting with lymphoproliferation and autoimmune cytopenia(s) remain without a molecular diagnosis

One hundred and sixty-three patients from nine European countries (*Online Supplementary Table S1*) fulfilled the inclusion criteria of splenomegaly or chronic lymphadenopathy and presumed or proven autoimmune cytopenia(s). Autoantibodies against red blood cells, platelets or granulocytes were detectable in 57 (35%), the other patients were either not tested or negative, but diagnosed with probable autoimmune cytopenia according to clinical judgment. Apoptosis testing was performed in 157 patients and was abnormal in 39 (24%). Among these 39 patients, 28 had germline *FAS* mutations (ALPS-FAS) and 11 had no germline mutation in *FAS*, *FASL*, *CASP8* or *CASP10* (ALPS-U). Three of these 11 patients were investigated for a somatic *FAS* mutation, which was found in two of them. The other eight patients were not investigated for somatic mutations either because defective apoptosis was a familial trait (n=3) or because the patients' material was not sufficient for sorting DNT (n=5). Among 113 patients with normal apoptosis, 53 were investigated for mutations in sorted DNT cells (n=46) or in germline DNA (n=7). Of these, four patients had a somatic *FAS* mutation (ALPS-sFAS), while 49 had no mutations in any of the four genes (designated "ALPS-phenotype sequenced": ALPS-ph-s). Eleven of the 163 patients did not have interpretable apoptosis tests. Six of these 11 patients were sequenced for germline *FAS* mutations, which were detectable in four. Among the 65 patients who did not undergo genetic analysis (designated "ALPS-phenotype not sequenced": ALPS-ph-ns), 11/61 (18%) had DNT cells <1%, precluding reliable exclusion of somatic mutations and for the remaining patients, the physicians pursued other diagnoses so that additional samples were not sent for DNT sorting.

Overall, in our cohort of 163 patients with splenomegaly or chronic lymphadenopathy and presumed or proven autoimmune cytopenia(s), 32 were diagnosed with ALPS-FAS and six with ALPS-sFAS. In seven patients, a molecular diagnosis other than ALPS was eventually established, including one patient each with DiGeorge syndrome, ICOS deficiency, LRBA deficiency, STIM-1 deficiency, X-linked lymphoproliferative syndrome-2, X-linked chronic granulomatous disease and RAS-associated autoimmune leukoproliferative disease. In addition, 19 of 36 patients with lymphoproliferation, autoimmune cytopenia(s) and hypogammaglobulinemia were clinically classified as having common variable immunodeficiency. No disease-causing mutations were found in *CASP8* or *CASP10* (*Online Supplementary Figure S1*).

Biomarker profiling in patients with lymphoproliferation and autoimmune cytopenia(s)

We then analyzed the distribution of values for 11 biomarkers in the five categories of patients (detailed information on missing values is provided in *Online Supplementary Table S2*). Similar to a previous retrospective report on a large cohort of patients with lymphoproliferation and more than 1% DNT cells,[12] sFASL, IL-10, vitamin B12 and DNT levels were increased in ALPS-FAS and ALPS-sFAS patients when compared to ALPS-U and ALPS-phenotype patients (Figure 1 and *Online Supplementary Figure S2*). We also studied a number of additional biomarkers that were previously reported to be altered in ALPS. Their distribution between ALPS-FAS/sFAS and control groups is shown in *Online Supplementary Figure S2*. sCD25 was elevated in most ALPS-FAS patients, but also in all other groups of patients, indicating an association with lymphoproliferation and autoimmunity in general rather than with ALPS-FAS in particular. Serum HDL and APO-A1 were decreased in most ALPS-FAS patients, but also in many other patients. B220 expression on DNT cells was increased in only 12/20 of ALPS-FAS patients, 3/5 ALPS-sFAS patients but also in 2/6 of ALPS-U and 10/36 of sequenced and 5/40 of not sequenced ALPS-phenotype patients. When compared to age-related normal values, MZB and SMB were reduced in 90% and 86% of ALPS-FAS patients, respectively, and in 100% and 83% of ALPS-sFAS patients, compared to 56% and 78% of ALPS-U, 63% and 88% of sequenced and 70% and 89% of not sequenced ALPS phenotype patients. IgG levels were above normal in 15/27 (56%) and below normal in 3/27 (11%) of ALPS-FAS patients. Elevated IgG was also observed in 17% of sequenced ALPS-phenotype patients, while 27% of not sequenced ALPS-phenotype patients had IgG levels below the age-related normal range. Biomarker values in patients with defined molecular diagnoses other than ALPS are shown in *Online Supplementary Table S3*.

To investigate the potential of the single biomarkers to diagnose a FAS mutation, we determined AUC values for each biomarker (Table 2) and ROC curves (*Online Supplementary Figure S3*) for the 98 sequenced patients.

Table 1. Description and classification of patients included in our study.

Patient category	N.	Description
ALPS-FAS	32	Patients with germline mutation in *FAS*
ALPS-sFAS	6	Patients with somatic mutation in *FAS*
ALPS-U	9	Patients with defective *FAS*-mediated apoptosis *in vitro* and no mutation in *FAS*, *FASL*, or *CASP10*
ALPS-ph-s	51	Patients with normal or unknown *FAS*-mediated apoptosis *in vitro* and no mutation in *FAS*, *FASL*, or *CASP10*
ALPS-ph-ns*	65	Patients with normal or unknown *FAS*-mediated apoptosis *in vitro* in whom *FAS* was not sequenced

** Patients not included in the statistical analysis.*

©Ferrata Storti Foundation 2013

The AUC was 0.92 for vitamin B12, 0.91 for sFASL and 0.86 for IL-10, which were the best discriminators. The value for DNT (0.74) was comparable to those for B220, IgG, sCD25, HDL, APO-A1 or MZB, all of which were in the range of 0.69-0.76. Thus, in our cohort of patients with splenomegaly or lymphadenopathy and autoimmune cytopenia(s), vitamin B12 and sFASL were the best predictors of a *FAS* mutation and significantly better than DNT levels (P=0.006 and P=0.012, respectively). Comparison to the available published AUC values in the NIH study showed that sFASL performed in a similar way to the NIH study, DNT slightly worse while vitamin B12 and IL-10 performed significantly better (P<0.01 and P=0.04, respectively).

Positive and negative predictive values of biomarker combinations for the diagnosis of ALPS-FAS

Subsequently, we analyzed the predictive values of the biomarkers studied. In order to facilitate comparison between the studies, we first used cut-off values and combinations suggested by Caminha *et al.*[12] (Table 3A). In our cohort with a prevalence of *FAS* mutations of 39%, vitamin B12 (>1500 pg/mL), sFASL (>300 pg/mL) or IL-10 (>40 pg/mL) alone identified between 27%, 44% and 43% of the patients with predictive values of 87%, 81% and 73%, respectively. In contrast, DNT (>4%) only reached a PPV of 63%, although it identified a group of patients of similar size (34%). The PPV of DNT was significantly lower than that in the NIH study (P<0.001). Combination of vitamin B12 and sFASL led to a PPV of 95% and still identified a group of 25%. Combining DNT with vitamin B12 or sFASL also yielded PPV above 90%, but identified fewer patients (*Online Supplementary Table S4A*).

Looking at the prediction of the absence of a *FAS* mutation (Table 3A), sFASL (<200 pg/mL) had a negative predictive value (NPV) of 97% and identified 43% of all patients. Vitamin B12 (<1000 pg/mL) and IL-10 (<20 pg/mL) identified slightly larger groups and had NPV of 91%, which were significantly higher compared to those in the NIH study (P<0.001 for both). DNT (<2%) had a NPV above 70% and identified only a third of all patients. Combining sFASL and vitamin B12 identified a group of similar size and with a NPV of 100%. Combining DNT with sFASL and vitamin B12 identified fewer than 25% of the patients (*Online Supplementary Table S4B*).

©Ferrata Storti Foundation 2013

Table 2. Area under the curve (AUC) values for all tested biomarkers.

Parameter	AUC	95% CI	AUC (NIH)
Vitamin B12	0.92	0.87-0.98	0.76
sFASL	0.91	0.83-0.98	0.90
IL-10	0.86	0.78-0.94	0.77
sCD25	0.76	0.66-0.86	NA
B220	0.74	0.63-0.85	NA
IgG	0.71	0.61-0.87	NA
DNT	0.74	0.60-0.82	0.81
HDL	0.71	0.59-0.82	NA
APO-A1	0.69	0.57-0.81	NA
MZB	0.69	0.58-0.79	NA
SMB	0.54	0.42-0.66	NA

AUC values were calculated from receiver operator characteristic (ROC) curves and reflect the diagnostic values of the different parameters to predict the presence of a FAS *mutation. Parameters are listed in descending order with respect to the value of the AUC. Values obtained from the study by Caminha* et al.[12] *are shown for comparison. Patients in the ALPS-not sequenced group are not included. CI: confidence interval, NA: not available.*

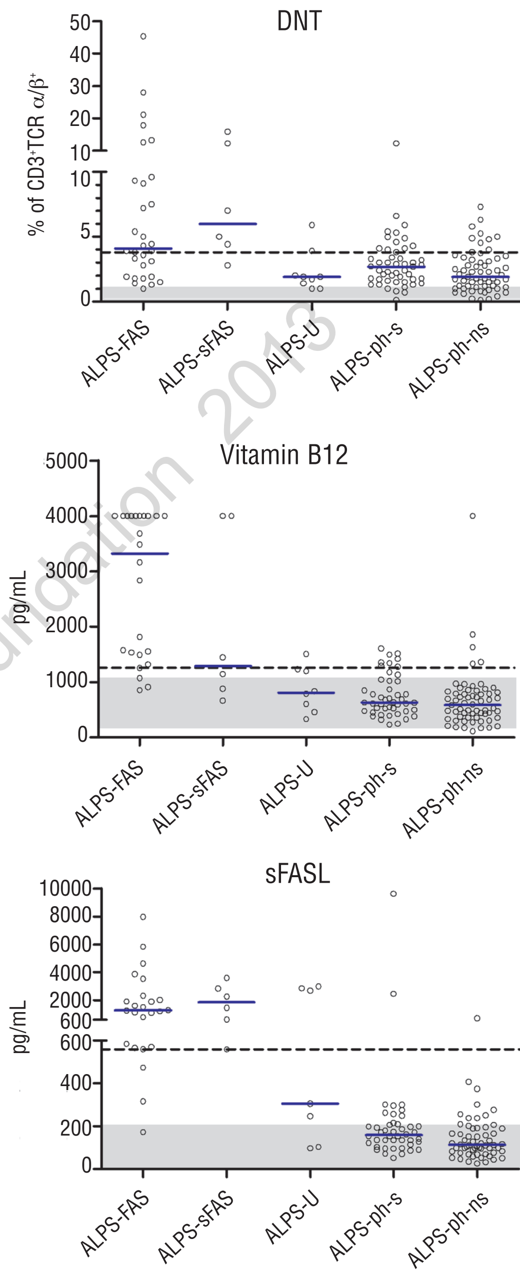

Figure 1. Biomarker values in the analyzed groups of patients. Values for DNT (CD4⁻CD8⁻ of CD3⁺TCRα/β⁺ T cells), vitamin B12 and sFASL are shown. The vertical blue bars denote median values. Shaded areas represent normal ranges. In the case of known age dependency (vitamin B12), the variable normal ranges cannot be depicted for the whole cohort in one plot; the range shown represents normal values for the most prevalent age group in our cohort (10-18 years). The dashed lines indicate the cut-offs identified in this study based on the prevalence of *FAS* mutations in our cohort as described in the *Methods* section. The upper limit of detection for vitamin B12 was 4000 pg/mL. Differences between the ALPS-FAS/sFAS and the other groups, examined with the Kruskal Wallis test, reached high statistical significance for all three parameters (P<0.001).

One of the problems of the cut-off values defined by Caminha *et al.*[12] was that they covered highly variable fractions of our population. For example, while 44% of our population had elevated sFASL levels above 300 pg/mL, only 27% had elevated vitamin B12 levels above 1500 pg/mL. In a second step, we therefore considered a different set of cut-off values that were chosen according to the prevalence of the *FAS* mutation in our cohort leading to identification of 40% of the patients as positive and 60% as negative (Table 3B). Using these cut-offs (sFASL 559 pg/mL, vitamin B12 1255 pg/mL, DNT 3.8%) the best biomarkers were sFASL (PPV 84% and NPV 94%) followed by vitamin B12 (PPV 73% and NPV 88%), while DNT performed much worse (PPV 61% and NPV 77%). Using biomarker combinations, vitamin B12 and sFASL above these cut-offs identified 29% of our population with a PPV of 92%, while both values below these cut-offs identified 49% of the population with a NPV of 97%. Other biomarker combinations did not perform better (*Online Supplementary Tables S4C* and *S4D*). Combining the more easily available biomarkers vitamin B12, HDL and APO-A1 yielded a PPV of 83% and identified 15% of our population. When these three values were below the cut-offs, 36% were identified with a NPV of 90%.

Using biomarker combinations to re-evaluate the results of genetic analysis

The high AUC values of the single markers and the high positive and negative predictive values observed for some combinations of markers suggested that these biomarkers can nearly separate the group of *FAS* mutation-positive from the group of *FAS* mutation-negative patients. This is illustrated by a scatter plot of vitamin B12 against sFASL with marking of the five considered categories of patients (Figure 2). ALPS-FAS/sFAS patients were well separated from patients with chronic lymphoproliferation and presumed or proven autoimmune cytopenia(s) of unknown molecular origin. Of note, in our original dataset there were five individuals among ALPS-U and ALPS-phenotype patients, who had a biomarker profile highly suggestive of a *FAS* mutation, with both vitamin B12 and sFASL values above the cut-offs of 1255 pg/mL and 559 pg/mL, respectively. We carefully re-evaluated the original genetic findings in these patients. In one patient, a misnaming of samples was uncovered and the patient was reclassified as having ALPS-sFAS. In another patient, two initial cell sortings did not yield sufficient numbers of DNT cells, and only when a third sample was sent, the genetic analysis could be completed revealing ALPS-sFAS. These two patients were reclassified prior to the analyses presented in this paper and are marked with arrows in Figure 2. One patient with familial ALPS-U had reduced FAS expression on T-cell subsets (*not shown*), suggesting a disturbed regulation of FAS expression. Two patients had their DNT re-analyzed but no mutation could be identified.

A biomarker-based probability calculator for selection or exclusion of patients with lymphoproliferation and autoimmunity to undergo FAS sequencing

As the best single markers were already highly predictive and the size of our population was limited, it was not possible to determine optimal scores combining several markers using methods such as logistic regression. To combine the information from single markers, we therefore used the naïve Bayes classification approach, which is based on the assumption of conditional independence of the markers given the *FAS* gene status, but is also known to be rather robust against violations of this assumption.[17,18] This approach makes use only of the means and standard deviations of each marker in *FAS* mutation-posi-

©Ferrata Storti Foundation 2013

Table 3. Positive predictive values (PPV) and negative predictive values (NPV) of selected biomarkers or their combinations for having a *FAS* mutation.

A

Parameter	Cut-off	Probability of having *FAS* mutation: Current study Prevalence (%)	Current study PPV (%)	NIH study * PPV (%)	Cut-off	Probability of not having *FAS* mutation: Current study Prevalence (%)	Current study NPV (%)	NIH study * NPV (%)
Vitamin B12	> 1500 pg/mL	27	87	87	< 1000 pg/mL	50	91	65
sFASL	> 300 pg/mL	44	81	88	< 200 pg/mL	43	97	92
IL-10	> 40 pg/mL	43	73	85	< 20 pg/mL	51	91	67
DNT	> 4 %	34	63	89	< 2 %	33	74	76
Vitamin B12 + sFASL	both above cut-off	25	95	NA	both below cut-off	33	100	NA

B

Parameter	Cut-off	Probability of having *FAS* mutation: Current study Prevalence (%)	Current study PPV (%)	NIH study * PPV (%)	Cut-off	Probability of not having *FAS* mutation: Current study Prevalence (%)	Current study NPV (%)	NIH study * NPV (%)
Vitamin B12	≥ 1255 pg/mL	39	73	NA	< 1255 pg/mL	61	88	NA
sFASL	≥ 559 pg/mL	40	84	NA	< 559 pg/mL	60	94	NA
IL-10	≥ 58 pg/mL	40	74	NA	< 58 pg/mL	60	87	NA
DNT	≥ 3.8 %	40	61	NA	< 3.8 %	60	77	NA
Vitamin B12 + sFASL	both above cut-off	29	92	NA	both below cut-off	49	97	NA

(A) Cut-off values were used as defined by Caminha et al. *(NIH study[12]). (B) Cut-offs values used were based on prevalence of* FAS *mutations in our cohort as described in the* Methods *section. Patients in the ALPS-not sequenced group are not included. NA: data not available, * data on prevalence unknown.*

tive and *FAS* mutation-negative patients and allows computation of the probability that a patient with lymphoproliferation and presumed or proven autoimmune cytopenia(s) carries a *FAS* mutation based on measurements for any set of markers. Based on this algorithm, we have created a web-based tool (Figure 3), in which clinicians can enter the values for vitamin B12, sFASL and IL-10 of their patients to estimate the probability of underlying *FAS* mutations (*http://www.alps.uni-freiburg.de/*). We included IL-10 to allow a more flexible use of the tool. In particular, it will enable retrospective validation of the tool with patients who did not have the two best biomarkers determined. It is important to note that calculated probabilities are only estimates which are clinically useful for selection of patients with high (>95%) *versus* low (<5%) probabilities of a *FAS* mutation. Values between 5% and 95% can only exclude high or low risk of a *FAS* mutation but do not represent reliable estimates of *FAS* mutation probability.

Although validation of the tool and diagnostic path need to be carried out on different data sets in the future, we analyzed the distribution of the individual probabilities according to this tool in our population in order to check whether the general robustness of the naïve Bayes classifier held in our situation. If not, this should have become visible by such an analysis. ALPS-U patients were excluded from this evaluation as their biomarker values do not fit into either of the ALPS-FAS or ALPS-phenotype groups. When the probability of *FAS* mutation was calculated using the three strongest predictors (vitamin B12, sFASL and IL-10), we observed that 35% of the study population had a high probability (>95%), of whom 83% actually had the *FAS* mutation, and 56% had a low probability (<5%) with only 2% carrying a *FAS* mutation.

Discussion

In this prospective study on patients referred for a diagnostic evaluation of chronic lymphoproliferation and presumed or proven autoimmune cytopenia(s), we evaluated 11 biomarkers in the diagnosis of ALPS. Our results suggest determination of vitamin B12 and sFASL with a subsequent decision on *FAS* sequencing as the most efficient and cost-effective diagnostic pathway to establish a molecular diagnosis. The positive and negative predictive values determined in this study were integrated into a simple web-based probability calculating tool that can support the decision whether or not to perform *FAS* sequencing based on any combination of values for the best predictive biomarkers: IL-10, sFASL and vitamin B12.

Lymphoproliferation and autoimmune cytopenia(s) are common manifestations of impaired immune regulation and are frequently associated with primary immunodeficiencies, including, but not limited to ALPS. When conceiving our study, we reasoned that a prospective evaluation of diagnostic laboratory parameters could best be performed in an unselected cohort referred for a combination of clinical symptoms rather than in patients preselected for biological markers such as DNT cells. This approach should provide the most relevant disease population for this particular clinical situation. However, this implies that our study does not provide information on patients referred for the evaluation of lymphoproliferation or autoimmune cytopenia alone or in combination with other autoimmune manifestations or with DNT elevation. In fact, during the time of the study, we also evaluated 23 other patients who did not fulfill our clinical entry criteria and identified *FAS* mutations in 12 (11 germline, 1 somatic). It is important to be aware of this conceptual difference when discussing our findings in relation to the landmark studies of Magerus-Chatinet *et al.*[10] and Caminha *et al.*[12]

Despite these differences in study concepts, we could confirm the high predictive value of vitamin B12, sFASL and IL-10 for the presence of *FAS* mutations in an independent, prospectively evaluated European cohort. Positive and negative predictive values were best for sFASL, IL-10 and vitamin B12 as single markers and best for vitamin B12 and sFASL as a biomarker combination. Obviously, positive and negative predictive values of biomarkers critically depend on the definition of cut-offs, but the cut-offs defined by Caminha *et al.*[12] and the cut-offs generated in our cohort yielded similar results. Of note, single biomarkers performed better in exclusion of *FAS* mutation in our cohort than in the study by Caminha *et al.*[12] Although the different sizes of the study populations may be a relevant factor, this is most probably related to

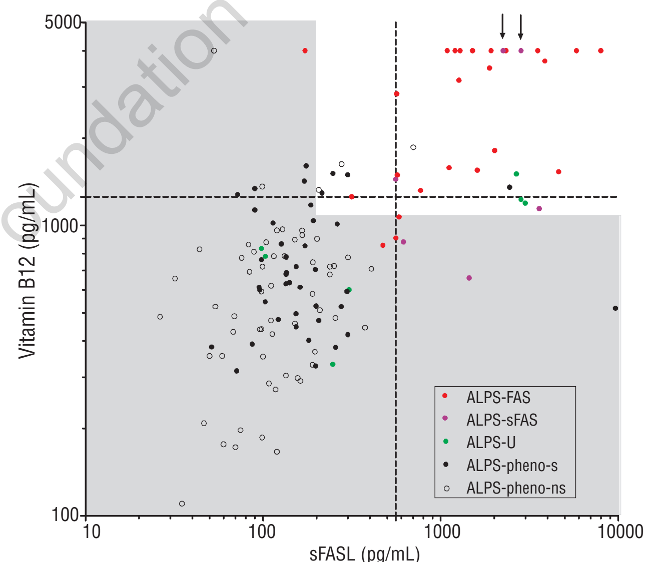

Figure 2. Scatter plot showing individual patients' values of vitamin B12 against sFASL for all investigated groups. Shaded areas represent normal values, the dashed lines depict the prevalence matched cut-offs (vitamin B12: 1255 pg/mL, sFASL: 559 pg/mL). In patients marked with arrows, somatic *FAS* mutations were detected upon re-analysis (see text for details).

Biomarkers:
Vitamin B12 (pg/mL)
IL-10 (pg/mL)
soluble FASL (pg/mL)
Reset
Calculate
The probability for *FAS* mutation in percent: %
For prospective evaluation of the calculator: Share Data

Figure 3. A web-based calculator of *FAS* mutation probability. Any number of the three most discriminative biomarkers, vitamin B12, sFASL and IL-10, can be entered to calculate the individual's probability of having a *FAS* mutation. (*http://www.alps.uni-freiburg.de*)

©Ferrata Storti Foundation

the different structure of the cohorts of patients with important implications for the calculation of predictive values. Thus, the percentage of patients with *FAS* mutations among all symptomatic patients was much higher (171 of 263; 65%) than in our study (38 of 98; 39%), which may be related to the fact that the retrospective study by Caminha *et al.*[12] was performed in a dedicated ALPS referral center. Moreover, the inclusion of healthy relatives in the control group of that study[12] represents a significant difference to our approach of only considering symptomatic patients.

The most relevant difference between the two studies was observed in the diagnostic value of DNT cells. When we initiated the study, DNT were defined as $CD4^-CD8^-$ of $CD3^+TCR\alpha/\beta^+$ cells among peripheral blood mononuclear cells using a threshold >1%. New guidelines recommend whole blood staining and thresholds of $CD4^-CD8^-$ $TCR\alpha/\beta^+$ of $CD3^+$ cells >2.5% or $CD4^-CD8^-TCR\alpha/\beta^+CD3^+$ of lymphocytes >1%. Following publication of these guidelines we performed both our initial as well as the recommended staining in all patients. DNT values determined using the suggested protocol[9] were slightly higher than those determined with our protocol. This has a limited impact on the results because it affected patients and controls equally. Since we did not have full blood stains for all recruited patients, we used the data obtained with our initial protocol for this analysis. DNT of $CD3^+/TCR\alpha/\beta^+$ were raised above 1% in 92% and above 2% in 69% of patients with disease control who did not carry *FAS* mutations. Since the proportion of disease controls was higher in our study, the predictive value of DNT determinations was much lower than in the study by Caminha *et al.*[12] Only when greater than 8%, were DNT levels reliably associated with a *FAS* mutation. In patients with chronic lymphoproliferation and autoimmune cytopenia(s), DNT levels therefore appear no more useful than the panel of additional biomarkers that were tested in our study including sCD25, serum lipids, memory B cells[13] and B220 expression on DNT cells.[14] Although characteristic alterations of these markers were observed in most patients with *FAS* mutations, they were similarly elevated or reduced in significant proportions of disease control groups, reducing their predictive value. It is relevant to note that the combination of the more easily available markers, vitamin B12, HDL and APO-A1, could exclude *FAS* mutation with a probability of 90% in 36% of our cohort (*Online Supplementary Table S4D*). However, the addition of any one of the other nine markers considered in our analyses to sFASL and vitamin B12 did not improve the positive or negative predictive values.

Interestingly, as observed previously,[12] some individuals among the ALPS-U and ALPS- phenotype patients had biomarker profiles highly suggestive of a *FAS* mutation. Given the high specificity of these markers, we hypothesized that they may have been misclassified or require more extensive genetic analysis. Indeed, two of these five patients were eventually reclassified as having ALPS-sFAS (Figure 2). In the other three, the characteristic biomarker profile could point to a more complex disturbance of FAS expression/function or to yet unknown defects in the FAS pathway; these patients are currently being analyzed for splice site and intronic *FAS* mutations, two have undergone whole-exome sequencing.

Another important issue in the diagnostic evaluation is the stability of biomarkers over time. It has been observed previously that sFASL, IL-10 and DNT cells decrease in response to immunosuppressive therapy.[10] We therefore performed additional analyses of our cohort, separating untreated patients (n=58) from treated patients (n=32) (*Online Supplementary Table S4*). Overall, positive and negative predictive values were comparable in untreated and treated patients. However, a clear decrease of the PPV of DNT was seen in treated patients. The variability of biomarkers in the long-term course of the disease and in response to different treatment regimen after initial diagnosis as well as their potential predictive impact on clinical outcome are additional relevant issues that need to be addressed in continued diagnostic and follow-up studies.

The identification of biomarkers which are specifically raised in patients with FAS mutations by Magerus-Chatinet *et al.*[10] and Caminha *et al.*[12] have very usefully extended the ALPS diagnostic panel and have been integrated into a revised diagnostic algorithm. This revised algorithm proposes germline sequencing in all patients with lymphoproliferation and raised DNT and makes use of biomarkers for subsequent selection of patients who require additional DNT sorting in search for somatic *FAS* mutations. Our results suggest that for the diagnostic evaluation of patients presenting with chronic lymphoproliferation and autoimmune cytopenia(s), an alternative diagnostic pathway can be followed. We propose initial determination of vitamin B12 and sFASL in order to decide who will need *FAS* sequencing. We still include analysis of DNT in the initial screening, but mainly to quantify the surface FAS expression of these cells, which can be helpful to identify patients with somatic second-hit mutations.[19,20] The combination of vitamin B12 and sFASL helps to identify patients with germline mutations as well as those with somatic *FAS* mutations and possibly also patients with as yet unknown mutations in the FAS pathway ('ALPS by biomarkers'). Moreover, it can identify *FAS* mutations as highly unlikely in a large proportion of patients with lymphoproliferation and autoimmunity.

Based on these data, it is our current policy to enter vitamin B12 and sFASL values into the probability calculator and if the probability is <5% (low risk), we do not sequence *FAS*. If clinical suspicion remains in the subsequent clinical course, biomarkers are determined again, since they can vary. If the calculated probability is >95% (high risk), we proceed to germline *FAS* sequencing and, if this is negative, we sequence DNA from sorted DNT cells. In patients with a probability between 5% and 95%, we base the decision on *FAS* sequencing on DNT levels and apoptosis induction: if both are normal, we do not sequence *FAS*, but if either DNT levels are above 3.8% or apoptosis is defective or both we proceed to *FAS* sequencing from germline and/or DNT DNA. The results of applying this algorithm retrospectively to our cohort for estimation of robustness of the calculating tool are shown in *Online Supplementary Figure S4*. This sequential diagnostic approach must be validated prospectively in independent cohorts of patients. The online calculator tool developed in this study will contribute to this evaluation. Thus, our findings provide additional prospective information and can contribute to evolving consensus recommendations addressing diagnostic algorithms in patients with lymphoproliferation and autoimmune cytopenia(s).

The spectrum of other genetic diseases identified in our cohort reflects the broad differential diagnosis in patients

©Ferrata Storti Foundation

with chronic lymphoproliferation and autoimmune cytopenia(s). To date the majority of these patients remain without a genetic diagnosis. One important challenge for future studies is to better define a rational diagnostic approach to these patients. This includes algorithms to select those in whom *FASL*, *CASP8*, *CASP10* or *K/N-RAS* mutations must be looked for very thoroughly, both at germline and somatic levels.

Acknowledgments

We would like to thank the patients and their families for their participation. We also thank the technicians of the CCI advanced diagnostic unit and the study nurse Henrike Ritterbusch. We also gratefully acknowledge the assistance of Stefan Rusch, Daniel Fix and Gerhard Kindle in designing the database and Lukas Sättler and Dr. Martin Boeker in preparing the web-based calculator.

Funding

This study was supported by the Bundesministerium für Bildung und Forschung (BMBF 01 EO 0803 and 01 GM 1111B). Dr. A. Janda is a recipient of an unrestricted fellowship grant from the European Society for Immunodeficiencies (ESID) provided by Baxter.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Notarangelo LD, Gambineri E, Badolato R. Immunodeficiencies with autoimmune consequences. Adv Immunol. 2006;89:321-70.
2. Teachey DT. New advances in the diagnosis and treatment of autoimmune lymphoproliferative syndrome. Curr Opin Pediatr. 2012;24(1):1-8.
3. Fisher GH, Rosenberg FJ, Straus SE, Dale JK, Middleton LA, Lin AY, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell. 1995;81(6):935-46.
4. Rieux-Laucat F, Le Deist F, Hivroz C, Roberts IA, Debatin KM, Fischer A, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. Science. 1995;268(5215):1347-9.
5. Holzelova E, Vonarbourg C, Stolzenberg MC, Arkwright PD, Selz F, Prieur AM, et al. Autoimmune lymphoproliferative syndrome with somatic Fas mutations. N Engl J Med. 2004;351(14):1409-18.
6. Niemela JE, Lu L, Fleisher TA, Davis J, Caminha I, Natter M, et al. Somatic KRAS mutations associated with a human nonmalignant syndrome of autoimmunity and abnormal leukocyte homeostasis. Blood. 2011;117(10):2883-6.
7. Oliveira JB, Bidere N, Niemela JE, Zheng L, Sakai K, Nix CP, et al. NRAS mutation causes a human autoimmune lymphoproliferative syndrome. Proc Natl Acad Sci USA. 2007;104(21):8953-8.
8. Takagi M, Shinoda K, Piao J, Mitsuiki N, Matsuda K, Muramatsu H, et al. Autoimmune lymphoproliferative syndrome-like disease with somatic KRAS mutation. Blood. 2011;117(10):2887-90.
9. Oliveira JB, Bleesing JJ, Dianzani U, Fleisher TA, Jaffe ES, Lenardo MJ, et al. Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. Blood. 2010;116(14):e35-40.
10. Magerus-Chatinet A, Stolzenberg MC, Loffredo MS, Neven B, Schaffner C, Ducrot N, et al. FAS-L, IL-10, and double-negative CD4- CD8- TCR alpha/beta+ T cells are reliable markers of autoimmune lymphoproliferative syndrome (ALPS) associated with FAS loss of function. Blood. 2009;113(13):3027-30.
11. Dowdell KC, Niemela JE, Price S, Davis J, Hornung RL, Oliveira JB, et al. Somatic FAS mutations are common in patients with genetically undefined autoimmune lymphoproliferative syndrome. Blood. 2010;115(25):5164-9.
12. Caminha I, Fleisher TA, Hornung RL, Dale JK, Niemela JE, Price S, et al. Using biomarkers to predict the presence of FAS mutations in patients with features of the autoimmune lymphoproliferative syndrome. J Allergy Clin Immunol. 2010;125(4):946-9.
13. Rensing-Ehl A, Warnatz K, Fuchs S, Schlesier M, Salzer U, Draeger R, et al. Clinical and immunological overlap between autoimmune lymphoproliferative syndrome and common variable immunodeficiency. Clin Immunol. 2010;137(3):357-65.
14. Bleesing JJ, Brown MR, Dale JK, Straus SE, Lenardo MJ, Puck JM, et al. TcR-alpha/beta(+) CD4(-)CD8(-) T cells in humans with the autoimmune lymphoproliferative syndrome express a novel CD45 isoform that is analogous to murine B220 and represents a marker of altered O-glycan biosynthesis. Clin Immunol. 2001;100(3):314-24.
15. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics. 1988;44(3):837-45.
16. StataCorp. Stata Statistical Software. College Station, TX: StataCorp LP; 2011.
17. Bickel PJ, Levina E. Some theory of Fisher's linear discriminant function, `naive Bayes', and some alternatives when there are many more variables than observations. Bernoulli. 2004;10(6):989-1010.
18. Dudoit S, Fridlyand J, Speed TP. Comparison of discrimination methods for the classification of tumors using gene expression data. J Am Stat Assoc. 2002;97(457):77-87.
19. Kuehn HS, Caminha I, Niemela JE, Rao VK, Davis J, Fleisher TA, et al. FAS haploinsufficiency is a common disease mechanism in the human autoimmune lymphoproliferative syndrome. J Immunol. 2011;186(10):6035-43.
20. Magerus-Chatinet A, Neven B, Stolzenberg MC, Daussy C, Arkwright PD, Lanzarotti N, et al. Onset of autoimmune lymphoproliferative syndrome (ALPS) in humans as a consequence of genetic defect accumulation. J Clin Invest. 2011;121(1):106-12.

©Ferrata Storti Foundation