

### Evolution of disease activity and biomarkers on and off rapamycin in 28 patients with autoimmune lymphoproliferative syndrome

Chronic benign lymphoproliferation and autoimmune cytopenias are the main features requiring treatment in FAS mutant patients with autoimmune lymphoproliferative syndrome (ALPS).<sup>1,2</sup> Successful use of the mTOR inhibitor rapamycin was initially reported in the treatment of refractory cytopenia in 3 ALPS-FAS patients.<sup>3</sup> The remarkable efficacy as a second-line agent for this indication was confirmed in a recent prospective study including a further 9 ALPS-FAS patients.<sup>4</sup> Here, we analyze aspects of rapamycin therapy that have so far not been addressed including first- versus second-line therapy, comprehensive biomarker responses, and the consequences of stopping rapamycin by reporting our experience in 28 ALPS-FAS patients.

We performed a retrospective survey of ALPS patients enrolled into research protocols in Paris, France (DC2011-1338) and Freiburg, Germany (DRKS00000298). Patients were included if they fulfilled NIH diagnostic criteria of ALPS<sup>5</sup> with genetical confirmation, and had received rapamycin for more than 6 months. Lymphoproliferation was defined as enlarged

spleen or lymphadenopathy ( $\geq 2$  lymph nodes in  $\geq 2$  sites enlarged for  $\geq 3$  months). Autoimmune cytopenia required autoantibodies or documented response to immunosuppression. In patients not previously treated with steroids or immunosuppressive drugs, rapamycin was regarded as first-line therapy. IVIG was not considered immunosuppression. Rapamycin was initiated at 1-2.8 mg/m<sup>2</sup>/day (d) aiming for plasma levels of 2-10 ng/mL. Treatment responses were evaluated at 6-9 months and at last follow up. Complete remission (CR) was defined as normal blood counts with platelets over  $100 \times 10^9/L$ , absent splenomegaly (palpable  $<2$  cm) and lymphadenopathy and cessation of immunosuppression including steroids. Partial remission (PR) was defined as persistent symptoms but a 50% or greater decrease in spleen size and/or in a reference lymph node and cessation of steroids without relapse of cytopenia.

Of 28 patients, 19 had heterozygous germline *TNFRSF6* mutations, one had a homozygous mutation; 8 had somatic mutations (*Online Supplementary Table S1*). Median age at disease onset was 4 (0-26) years (Table 1). The indication for rapamycin was lymphoproliferation in 8, lymphoproliferation and autoimmunity in 18, and autoimmunity in 2 patients. Overall, 19 patients had autoimmune cytopenia [16 in 1 lineage (mostly AIHA), 2 in 2 lineages, 1 in 3 lineages]. Before rapamycin, 7

Table 1. Treatment history and rapamycin response of individual patients.

#### A) Pretreated patients with second-line rapamycin treatment

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
age of onset	1m	12m	5y	4m	2m	12y	11y	9y	7m	10m	3.4y	14y	3y	2m	4y	18m	6m	6m	4y
age start IS	1m	7y	7y	4m	2m	12y	11y	9y	3y	10m	3.4y	14y	3y	2m	4y	18m	4y	16y	8y
steroids																			
MMF																			
6MP																			
AZA																			
IVIG																			
HSCT																			
anti-CD20																			
others																			
response <sup>1</sup>			tox							tox					tox	tox	tox		tox
age start rapa [y]	13	16	13	6	5	13	14	10	6	1.9	4	14	5	7	5	1.6	6	17	14
rapa response (6 m) <sup>1</sup>																			
rapa response (last FU) <sup>1</sup>																			
rapa side effects <sup>2</sup>																			

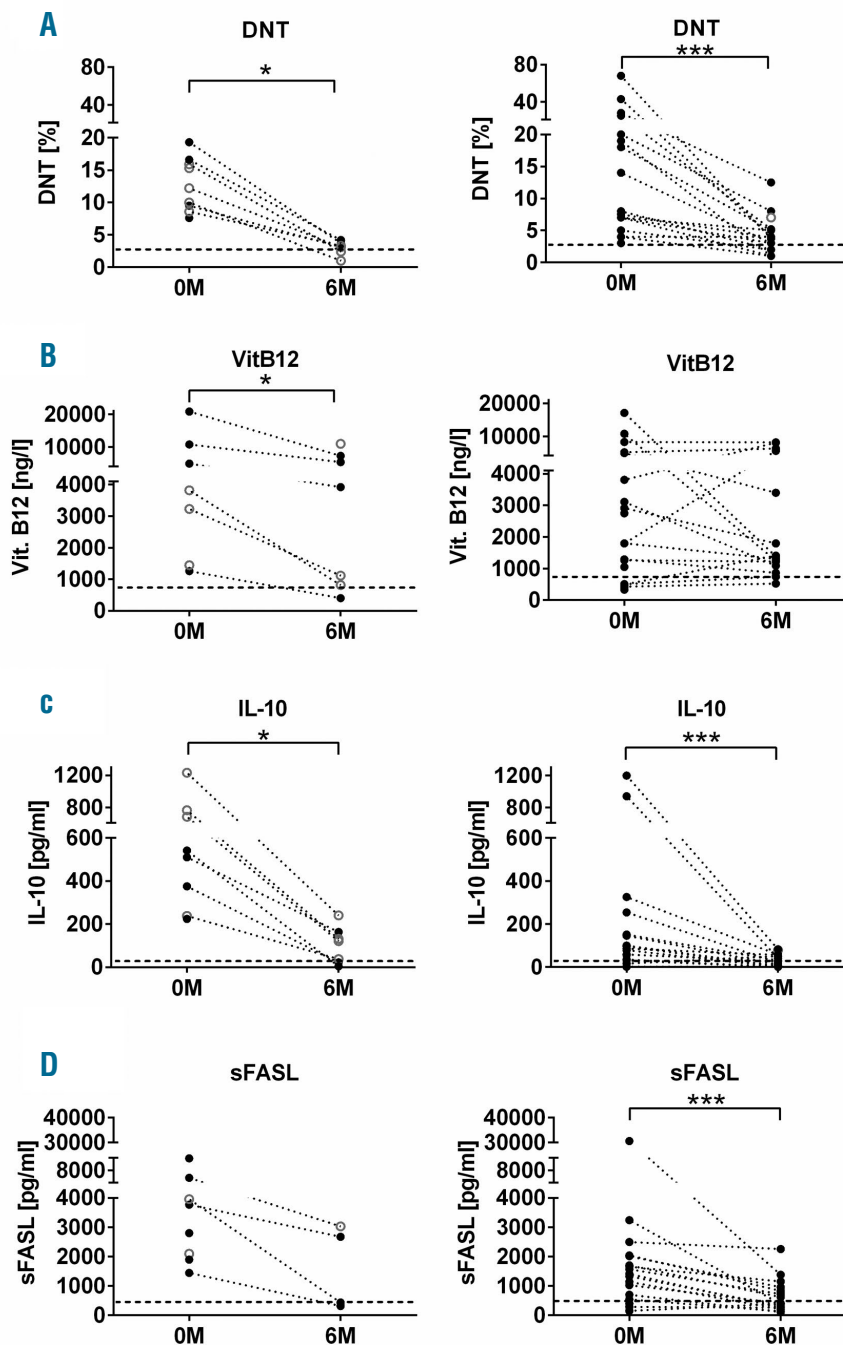
#### B) Patients with first-line rapamycin treatment

Patient	20	21	22	23	24	25	26	27	28
age of onset	3.7y	2.3y	12m	10m	2.0y	8m	22m	2.5y	26y
age start rapa	4y	4y	15y	12m	14y	12m	6y	8y	26y
rapa response (6 m) <sup>1</sup>									
rapa response (last FU) <sup>1</sup>			off						off
rapa side effects <sup>2</sup>									

<sup>1</sup>Patients ns.1-19 were pre-treated as indicated by gray boxes. <sup>2</sup>Response to treatment: white: no response; gray: partial remission; black: complete remission. "tox": necessity to stop the drug due to side effects. <sup>3</sup>Side effects of rapamycin: white: none; gray: moderate; black: requiring stop. Age in years (y) or months (m); IS: immunosuppressive treatment; MMF: mycophenolate mofetil; MP: 6-mercaptopurine; AZA: azathioprine; IVIG: intravenous IgG; HSCT: hematopoietic stem cell transplantation; anti-CD20 monoclonal antibodies; rapa: rapamycin; FU: follow up.

patients had no previous therapy, 2 had received IVIG. In these 9 patients, rapamycin was considered first-line therapy (Table 1). It was initiated as monotherapy in 8 patients and in P21 together with steroids and IVIG. The remaining 19 patients had received up to 6 lines of previous therapies for a median of 3.7 (0.3-13) years. Two patients had undergone hematopoietic stem cell transplantation. Both had disease relapses due to graft rejection (P1) and mixed chimerism (P14). In these patients, rapamycin was considered second-line therapy (Table 1). At initiation of rapamycin, 3 of 19 had no response to previous treatment, 13 were in PR and 3 had responded, but had side effects (Table 1). Except for 2 cases, ongoing therapy was maintained (steroids n=9, steroids+6MP n=2, 6MP n=4, AZA n=3, MMF n=1). Rapamycin was

introduced at a median dose of 2 (1-2.8) mg/m<sup>2</sup>. After 6-9 months of treatment, 22 patients (79%) were in CR and 6 (21%) in PR. Rapamycin add-on therapy led to CR in 94% (17 of 18). The other immunosuppressive drugs were discontinued in all patients within six months, except for one with PR (P13). In that patient, who had presented with severe AIHA, rapamycin resolved the splenomegaly and stabilized, but did not fully control AIHA. Nevertheless, corticosteroids could be terminated. First-line rapamycin induced CR in 4 and PR in 5 patients (Table 1 and *Online Supplementary Figure S1*). The incomplete treatment response was associated with documented poor compliance in 2 patients (P22 and P28). The other 3 PR patients were satisfied with the response achieved and no dose escalation or alternative therapies



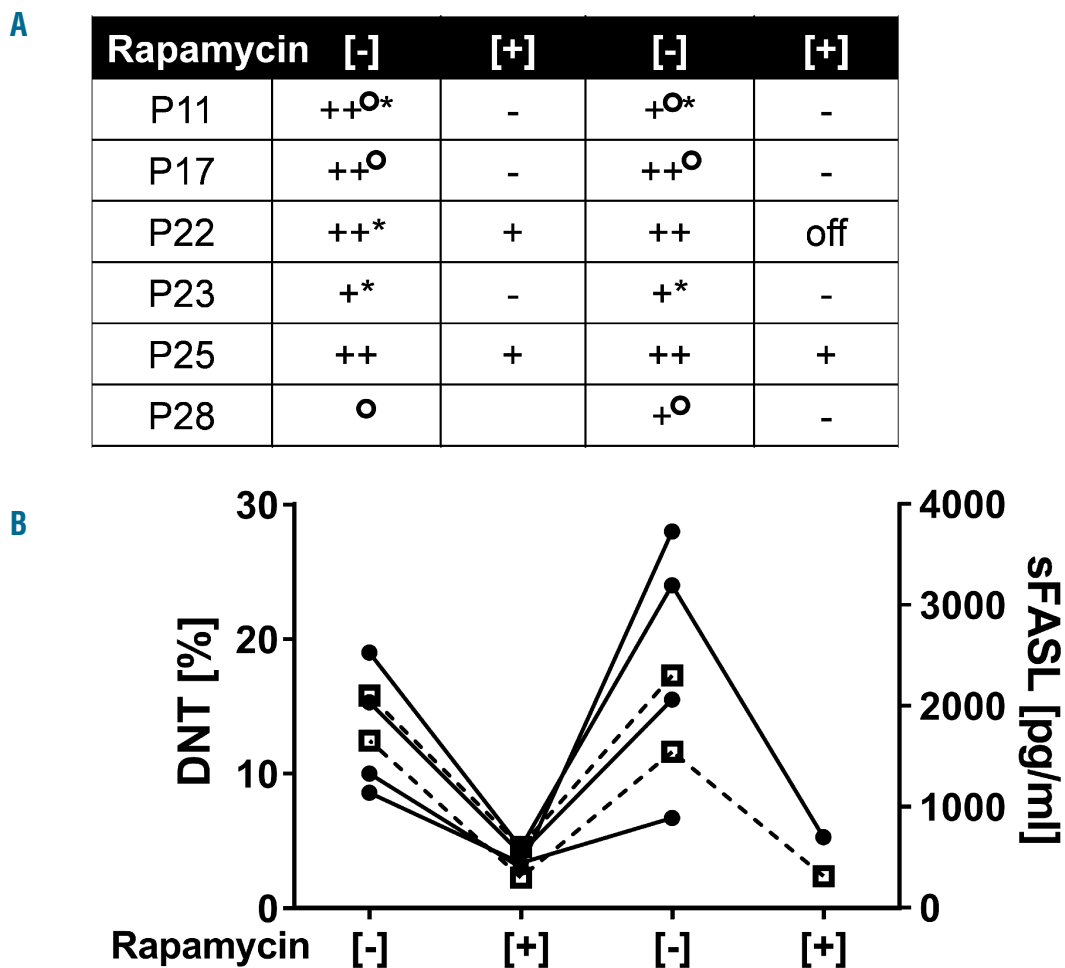
**Figure 1.** Biomarker responses at six months in patients receiving rapamycin as first-line or second-line treatment. The percentage of DNT cells among CD3<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> lymphocytes (A), the serum levels of VitB12 (B), IL-10 (C) and sFASL (D) were determined before rapamycin therapy and 6-8 months after initiation of treatment in patients who received rapamycin as first-line therapy (left panels) or after prior immunosuppressive treatment (right panels). Patients with complete remission are depicted in black, partial remissions are indicated by open gray circles. Analyses were performed using PRISM-software (GraphPad software, San Diego, USA). Populations were compared using the Wilcoxon matched-pairs signed rank *t*-test. *P*<0.05 was considered significant. \**P*<0.05; \*\*\**P*<0.001.

were attempted. Importantly, rapid stabilization of cytopenias was achieved among all 5 patients who received rapamycin as first-line therapy for AIHA or ITP. At last follow up, 26 patients were still on rapamycin. P28 developed IgG4-related disease while on rapamycin and it was decided to stop treatment, P22 stopped because of feeling “unwell” on the medication. Median treatment duration was 2.8 (0.5-5.6) years. All 23 patients with initial CR maintained CR (Table 1). Rapamycin dose was reduced in 14 of 22 patients where this information was available. At last follow up, serum levels were available in 10 patients and CR was maintained in all 6 patients at levels between 2-5 ng/mL.

As described,<sup>3,6</sup> DNT cells rapidly decreased upon rapamycin treatment, but only 7 of 25 patients (33%) reached normal percentages (Figure 1A). IL-10 levels decreased, but remained more than 20 pg/mL in 14 of 23 patients (61%) (Figure 1C). sFASL decreased, but only 2 of 28 patients reached normal values (Figure 1D). Significant biomarker decreases were observed in both first- and second-line treated patients. VitB12 levels remained elevated in 16 of 23 (69%) patients. While 6 of 19 patients with second-line treatment showed a drop in VitB12 levels, 7 of 19 showed an increase (Figure 1B). All

patients with rapamycin first-line treatment showed a decrease (Figure 1B). Among 22 patients in CR, 2 had complete normalization and 5 had close to normal values for DNT cells, IL-10 and sFASL, while 15 patients still had elevated biomarkers. There was no statistical difference in biomarker responses between PR and CR patients. Thirteen patients experienced mild adverse effects, including mouth ulcers (n=4), mild proteinuria (n=2), increased blood pressure (n=2), and transient skin rash, *Helicobacter Pylori* associated gastritis, transient liver enzyme elevation concomitant to herpes zoster and EBV infection in one patient each. In 6 patients, rapamycin was temporarily stopped due to noncompliance (n=3), complete remission (n=2) or elevated blood pressure (n=1). All 6 had rapid relapse of lymphoproliferation and 2 relapsed with autoimmune cytopenias, accompanied by rapid re-augmentation of biomarkers (Figure 2A and B). Five patients restarted treatment. All had the same response as during the first treatment episode (Figure 2A).

Our results provide novel, practically relevant insights on rapamycin therapy in ALPS-FAS based on the pioneering original observations by Teachey *et al.*<sup>3,7</sup> First, we confirm the efficacy and safety of rapamycin by reporting 28



**Figure 2. Effect of treatment discontinuation.** Summary of observations in 6 patients who discontinued rapamycin treatment. (A) Status of rapamycin treatment is indicated by [-]/[+]. The spleen size is indicated as follows: - not palpable; + palpable but <5 cm; ++ >10 cm or up to umbilicus. <sup>o</sup> Additional lymphadenopathy. \*Cytopenia. (B) Percentages of DNT cells (left y-axis, black circles) and sFASL levels (right y-axis, open boxes/broken lines) at different stages of therapy.

additional ALPS-FAS patients, all of whom responded to treatment. Our results also confirm the good tolerability within the treatment period of up to six years. Nevertheless, long-term side effects of rapamycin can be relevant and careful monitoring is required. Second, we present the first data on ALPS-FAS patients receiving rapamycin as first-line therapy. We were not only able to stop all other treatments in patients who had received several lines of prior therapy, but we also achieved excellent responses using rapamycin as the first and single agent. This included rapid control of cytopenia and lymphoproliferative manifestations. Nevertheless, immediate stabilization of autoimmune cytopenia cannot be expected and may require initial concomitant treatment. We propose to consider rapamycin as a first-line treatment in ALPS-FAS patients.

In genetically proven cases, there is a clear biological rationale for rapamycin. Lymphoproliferation in ALPS is not just due to an accumulation of DNT cells that cannot die, but these cells and their single positive precursor cells<sup>8</sup> are highly proliferative *in vivo*.<sup>6,9</sup> This proliferative activity is associated with hyperactive mTOR signaling.<sup>6</sup> Blocking proliferative activity and induction of apoptosis in DNT cells and their precursors are two non-mutually exclusive explanations for the impressive effect of rapamycin on lymphoproliferation.<sup>6,7</sup> The rapid relapse of disease after stopping treatment indicates that the cells giving rise to DNT cells are either not fully eliminated or are rapidly regenerated once targeted mTOR inhibition is stopped. How rapamycin abrogates autoimmune manifestations remains unclear. Fas deficient B cells escape germinal center selection and undergo enhanced somatic hypermutation.<sup>10</sup> Rapamycin could either directly affect B-cell signaling or survival and/or it could indirectly influence germinal center function by decreasing DNT and IL-10. While mTOR inhibition represents targeted molecular treatment for ALPS, this is less clear in patients with autoimmunity and lymphoproliferation in the context of other diseases clinically resembling ALPS-FAS. Although there are other immunodeficiencies characterized by mTOR activation (eg. activated PI3K delta syndrome<sup>11</sup>), the rapamycin response in these diseases is much more variable.<sup>4</sup> At present, we therefore advocate first-line rapamycin only in ALPS-FAS patients. Third, we present new observations relevant for the guidance of long-term therapy. Rapamycin levels less than 5 ng/mL were sufficient to maintain disease control in most patients. However, stopping rapamycin was associated with rapid relapse in all cases. Based on these observations, we currently start with 2 mg/m<sup>2</sup>/d and adapt this up to 10 ng/mL. Once full remission has been achieved, we titrate down to 2-5 ng/mL. Because at least the lymphoproliferative manifestations tend to attenuate with age, the necessity of life-long treatment remains unclear. Another open issue of long-term treatment is whether rapamycin reduces or increases the risk of lymphoma. While biomarkers have proven useful for establishing a diagnosis of ALPS-FAS,<sup>12-14</sup> their value in guiding therapy appears limited. Although a significant decrease was observed, CR was accompanied by biomarker normalization in no more than one-third of the patients. In summary, our results further establish rapamycin as an excellent targeted therapy for ALPS-FAS patients and provide support for its use as a first-line agent in this disease.

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