Protocol with Statistical Analysis Plan Cover Page:

Official Title:	A Randomized Trial of Sirolimus (Rapamune®) for Relapse Prevention in Patients with Severe Aplastic Anemia Responsive to Immunosuppressive Therapy
NCT number:	NCT02979873
Document Type:	Protocol with Statistical Analysis Plan (SAP)
Document Date:	June 9, 2022

CLINICAL RESEARCH PROJECT

Protocol #: 17-H-0019
IND: Exempt

Title: A Randomized Trial of Sirolimus (Rapamune®) for Relapse Prevention in Patients with Severe Aplastic Anemia Responsive to Immunosuppressive Therapy

Short Title: Sirolimus in Aplastic Anemia

Keywords: Sirolimus, Rapamune®, SAA, Rapamycin, Relapse Prevention, Immune Tolerance

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Subjects of Study:

Number:	**Sex**:	**Age-Range**:
54 per arm	Either	≥ 2 years and weight > 12 kg

Total: 118 to allow for replacement of non-evaluable subjects

Project Involves Ionizing Radiation?	No
Off-Site Project?	No
Multi-center trial?	No
DSMB Involvement?	Yes

PRECIS

- Most acquired aplastic anemia ensues from immune-mediated destruction of hematopoietic stem and progenitor cells (Young, 2013).
- Immunosuppression is the definitive treatment of patients with acquired aplastic anemia who are not candidates for immediate hematopoietic stem cell transplantation.
- Horse ATG combined with the calcineurin inhibitor, cyclosporine (CsA), remains standard as first-line immunosuppressive therapy (IST).
- Hematologic responses to transfusion independence occur in about two thirds of patients with standard IST and in 80-90% of patients treated with IST in combination with the growth factor eltrombopag.
- About 30% to 40% of patients relapse after discontinuation of cyclosporine. Many achieve disease control after the reinitiation of CSA, but remain CSA dependent indefinitely.
- Evidence from mouse models of bone marrow failure indicates that conversion from cyclosporine to the mTOR inhibitor, sirolimus (SRL), results in immune tolerance which can endure the eventual withdrawal of SRL.
- We hypothesize that CSA to SRL conversion will significantly decrease the relapse rate after immunosuppressive therapy for acquired aplastic anemia.
- This study will investigate the safety and efficacy of SRL for preventing relapse in patients previously treated with IST who remain on CSA. The primary endpoint is rate of relapse at 2 years following conversion from CSA to SRL, versus stopping CSA.
- Biological sampling of peripheral blood and bone marrow aspirates during treatment will be used to investigate changes to lymphocyte phenotypes and cytokine profiles.

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1.0 Objectives

1.1 Primary objectives

- To determine if the rate of relapse at 24 months after CSA discontinuation can be improved by conversion to sirolimus in severe aplastic anemia patients who have responded to IST.

1.2 Secondary objectives

- Safety and tolerability of sirolimus.
- Time to relapse, and rate of relapse at any time assessed before and after 24 months.
- Rate of clonal evolution to new cytogenetic abnormalities, myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), or rate of expansion of paroxysmal nocturnal hemoglobinuria clones.
- Explore the biological effects of sirolimus on lymphocyte and cytokine profiles.

2.0 Background and Scientific Justification

2.1 Acquired aplastic anemia

Severe aplastic anemia (SAA) is a bone marrow failure syndrome characterized by pancytopenia and bone marrow hypo-cellularity. Since all cell lines are affected, the etiology of this disease was early on recognized to be at the level of the pluripotent hematopoietic stem and progenitor cells. Indeed, hematopoietic stem cell transplantation (HSCT) is curative but is an option only for younger patients who have histocompatible donors. Clues to an immune-mediated etiology SAA came from reports of HSCT patients who failed to engraft after immunosuppressive conditioning yet who achieved autologous recovery with hematological remission(Gmur et al., 1979; Jeannet et al., 1974; Mathe et al., 1970; Sensenbrenner et al., 1977; Speck et al., 1976; Thomas et al., 1976). Clinical trials of immunosuppressive therapies with and without HSCT(Bukowski et al., 1978; Doney et al., 1981; Speck et al., 1978; Speck et al., 1981) found that immunosuppressive therapy alone was efficacious at least in a subset of patients. Further evidence for an immune-mediated etiology extrinsic to hematopoietic stem and progenitor cells came from syngeneic HSCTs without immunosuppressive conditioning (Appelbaum et al., 1980; Lu, 1981). In the study by Appelbaum et al 11 of 22 syngeneic HSCTs without immunosuppression failed to engraft, arguing against a quantitative HSC deficiency. All 6 of the 11 patients, who subsequently underwent repeat syngeneic HSCT with immunosuppressive conditioning, experienced complete hematological recovery. Interferon-γ, an important mediator of hematopoietic suppression was noted to be elevated in the sera of patients (Nakao et al., 1992; Nistico and Young, 1994; Young, 1986). Specific populations of effector T-cells were elevated and localized to the bone marrow (Zoumbos et al., 1984, Maciejewski 1994). High-resolution analyses of the complementarity-determining region 3 (CD3) of the β-chain (VB) T-cell receptor (TCR) in SAA patients (Kook et al., 2002; Zeng et al., 1999) showed non-random skewing, suggestive of oligoclonal T-cell proliferation. In SAA patients who responded to immunosuppressive therapy clonal predominance was no longer evident.

Therapy for SAA consists of either allogeneic hematopoietic stem cell transplantation (HSCT) or immunosuppressive therapy. Matched-sibling HSCT using bone marrow as the stem cell source has been the preferred first therapy in children and young adults (up to the age of 40 years) diagnosed with SAA. In patients older than 40 years, transplantation-related mortality and morbidity increase. With the insight that SAA is immune-mediated, older patients and in those without matched histocompatible donors are offered immunosuppressive therapies. The combination immunosuppressive regimen of horse anti-thymocyte globulin with cyclosporine has shown better response than monotherapies. The simultaneous addition of mycophenolate mofetil or sirolimus did not improve hematological response or decrease clonal progression or relapse (Scheinberg et al., 2009). Alternatives to hATG/CsA include cyclophosphamide and alemtuzumab.

While early reports suggested that treatment with cyclophosphamide resulted in lower rates of relapse and clonal evolution, this was not seen in randomized studies; infectious complications resulting from prolonged neutropenia were higher. Alemtuzumab was shown to be efficacious in the refractory setting. In recent years clinical progress has focused on the addition of stem cell growth factor (Olnes et al., 2012). The introduction of the TPO mimetic eltrombopag to immunosuppressive regimens containing horse anti-thymocyte globulin (hATG) and cyclosporine (CsA), has resulted in very high hematological response rates – 92% OR at 6 months, in the last cohort of patients on protocol 12-H-0150.

In patients who respond to immunosuppressive therapy with hATG and CsA, CsA is often continued for 6 months. In our eltrombopag added to standard immunosuppressive therapy protocol, CsA is tapered at 6 months and discontinued after 2 years. Historically approximately 30% to 40% of patients relapse after withdrawal of all immunosuppression. Some of these patients become dependent on continued immunosuppression for maintenance of adequate cell line counts. For the purposes of this protocol we shall define patients as having ***relapsed***, if, after initial hematological response to IST, their cell line counts either (i) decline to a degree where they again meet criteria for severe aplastic anemia or (ii) require continued immunosuppression to maintain adequate cell line counts.

2.2 Mechanism of action of cyclosporine, a calcineurin inhibitor

Calcineurin inhibitors (CNI) almost always as cyclosporine remain the standard of care in maintenance immunosuppressive therapies for aplastic anemia. The intracellular processes that underlie the action of CNIs are reasonably well understood (Azzi et al., 2013; Shevach, 1985). When an antigen-presenting cell presents cognate antigen to a T cell receptor (TCR), TCR activation results in increased intracellular Ca^{2+} concentration. This activates the Ca^{2+}-dependent phosphatase calcineurin, which dephosphorylates inactive cytosolic Nuclear Factor of Activated T cells (NF-AT). Activated NFAT translocate to the nucleus where it associates with other nuclear components leading to the transcription of genes encoding cytokines (IL-2, TNF-α, IL-3, IL-4, CD40L, GM-CSF, IFN-γ). CNIs bind intracellular immunophilins and interfere with activation of calcineurin, resulting in reduced expression of IL-2 and concomitantly decreased T lymphocyte activation and proliferation. More recent studies have shown that CNIs decreased proliferation of both effector as well as regulatory T-cells (Miroux et al., 2012). There are indications that the use of CNIs is deleterious to potential tolerogenic therapeutic strategies.

CNI have numerous adverse effects, which make their long-term use problematic. Direct toxic effects on renal tubular epithelial cells, chronic CNI use can cause tubulointerstitial fibrosis and focal glomerulosclerosis. Hypertension, secondary to renal vasoconstriction and Na^{+} retention, is a common cardiovascular adverse effect. CNI-induced metabolic abnormalities include diabetes mellitus and bone loss. Thrombotic microangiopathy, secondary to CNI-related secretion of ADAMTS13 is a relatively infrequent complication (4.9 per 1000 patient-years). CNI-related neurotoxicities include tremor, headache and hyperesthesias. Chronic immunosuppression with CNI raises the risk for CMV (15% - 25%), fungal and bacterial infections. CNI increase the risk of skin cancer 200-fold. CNI-related cosmetic issues can include gum hyperplasia, alopecia and hypertrichosis.

2.3 Mechanism of action of Sirolimus (Rapamune®)

Sirolimus inhibits T lymphocyte activation and proliferation that occurs in response to antigenic and cytokine (Interleukin [IL]-2, IL-4, and IL-15) stimulation by a mechanism that is distinct from that of other immunosuppressants. Sirolimus also inhibits antibody production. In cells, sirolimus binds to the immunophilin, FK Binding Protein-12 (FKBP-12), to generate an immunosuppressive complex. The sirolimus:FKBP-12 complex has no effect on calcineurin activity. This complex binds to and inhibits the activation of the mammalian Target of Rapamycin (mTOR), a key regulatory kinase. This inhibition suppresses cytokine-driven T-cell

proliferation, inhibiting the progression from the G1 to the S phase of the cell cycle.

mTOR is an important integrator of environmental cues critical for the regulation of T-cell activation, differentiation and function (Pollizzi and Powell, 2015). Inhibitors of mTOR are attractive immunomodulators due to their potential to induce adaptive tolerance and their relatively attractive side effect profiles. The importance of mTOR in regulating T-cell function was highlighted in studies of T-cell anergy. T_H1 cells that encounter antigen (Signal 1) in the absence of co-stimulation (Signal 2) become anergic, i.e. hypo-responsive upon re-challenge (Schwartz, 2003; Schwartz et al., 1989). mTOR integrates the two signals and determines the outcome of TCR engagement with regard to activation or anergy (Zheng et al., 2007). Experiments showed that activation of T cells in the presence of mTOR inhibition induced anergy even when cells received co-stimulation (Powell et al., 1999). Importantly, T-cells engineered to express a SRL-resistant mTOR were resistant to SRL-induced anergy (Zheng et al., 2007). Later studies revealed that inhibition of mTOR during activation not only resulted in T_H1 anergy, but it also promoted the expansion of/differentiation to regulatory T cells (Tregs) (Battaglia et al., 2005; Coenen et al., 2007; Kang et al., 2008; Kopf et al., 2007).

2.4 Sirolimus with CsA in Severe Aplastic Anemia

The complementary actions of CNI and mTOR inhibitors gave rise to the hope that better responses to immunotherapy might be achieved with the addition of mTOR inhibitors to CsA. In a prospective randomized study, 77 SAA patients were treated with standard immunosuppressive therapy for 6 months with or without *concurrent* sirolimus (Scheinberg et al., 2009). The study was closed to accrual after interim analysis of 30 evaluable patients did not show improved hematological response at 3 months in the sirolimus (SRL) arm. It is important to note that there was no evidence of increased toxicity with sirolimus on this protocol. Whether the addition of SRL improved relapse rates is not known.

2.5 Sirolimus in Non-myeloablative Hematopoietic Stem Cell Transplantation

mTOR inhibitors have seen successful application in hematopoietic stem cell transplants. In a mismatched mouse model using BALB/c donors and C57BL/6 recipients, recipients were conditioned with 2 Gy total body irradiation (TBI) and sirolimus (SRL) vs. CsA (+/- post-transplant cyclophosphamide). Mice treated with CsA rejected the transplant, whereas mice treated with SRL maintained stable mixed chimerism (Fitzhugh et al., 2013). Moreover, SRL-treated transplanted mice were shown to be functionally tolerant to the donor in *in vitro* and *in vivo* experiments.

SRL has been used in human non-myeloablative hematopoietic stem cell transplantation in patients with severe sickle cell disease with or without thalassemia (Hsieh et al., 2014). 26 of 30 patients (86%) achieved long-term stable donor engraftment without acute or chronic graft versus host disease. 15 engrafted patients discontinued immunosuppression with continued stable donor chimerism. Mean donor T-cell chimerism was 48% (95% CI: 34% - 62%) and without evidence of graft versus host disease. The results suggested functional tolerance between donor and recipient cells and demonstrated that mixed chimerism can be stable long-term.

2.6 CNI to mTORi conversion in solid organ transplants

In solid organ transplant patients who must remain on lifelong immunomodulation/suppression, the adverse effects of CNIs provide a strong motivation to identify an alternative therapy. One approach, which has been used in the solid organ transplant setting, is to replace the CNI with an mTOR inhibitor.

Several randomized, controlled trials have examined the efficacy after conversion from a calcineurin inhibitor to an mTOR inhibitor at time points as short as 10 days to more than 6 months in the setting of renal, hepatic and cardiac transplantation (Mulgaonkar and Kaufman,

2014).

SMART: Supporting Medication Adherence in Renal Transplantation (Guba et al., 2010) evaluated early conversion (10 – 24 days) from CsA to SRL after renal transplantation. Patients with low- to moderate-immunological risk (N = 141) were continued on CsA or converted to sirolimus (Guba et al., 2012); all patients were continued on mycophenolate mofetil (MMF) and corticosteroids. Patients randomized to the SRL arm were initiated on SRL at a loading dose of up to 0.1 mg/kg followed by 2 – 4 mg daily, initially targeting a whole-blood trough concentration of 8 – 12 ng/mL. On day 1 of conversion, the CsA dose was reduced by 50%; CsA was discontinued after an overlap of 3 days. At 3 months SRL was tapered to target a whole-blood trough concentration of 5 – 10 ng/mL. In the final analysis, the SRL arm showed better renal function than those in the CsA arm (64.5 mL/min/1.73 m^2 vs. 53.4 mL/min/1.73 m^2). There was no difference in patient survival, graft survival or incidence of biopsy-proven acute rejection.

CONCEPT: Efficacy on renal function of early conversion from cyclosporine to sirolimus three months after renal transplantation (Lebranchu et al., 2009) evaluated conversion from CsA to SRL 3 months after transplantation. 192 patients were randomized to CsA (n = 95) or SRL (n = 97) arms; all patients were continued on MMF and corticosteroids through month 8. At week 12 the SRL arm received a loading dose of 10 mg SRL daily for 2 consecutive days, followed by 6 mg SRL daily, adjusted to target a whole-blood trough concentration of 8 – 15 ng/mL, then 5 – 10 ng/mL after week 39; CsA was abruptly discontinued with the initiation of SRL. The primary endpoint was GFR at week 52, which was significantly better in the SRL arm (68.9 mL/min vs. 64.4 mL/min, p = 0.017). Patient and graft survival were not significantly different. Significantly more patients in the SRL arm reported aphthous stomatitis, diarrhea, acne and high triglyceride levels.

STN: Spare-the-Nephron (Weir et al., 2011) evaluated conversion to a SRL-based regimen at 30 – 180 days following transplantation. 299 patients were randomized to MMF and a CNI (tacrolimus, n = 120; cyclosporine, n = 31) versus MMF and SRL (n = 148); corticosteroids were continued. Patients on the SRL arm had CNI completely withdrawn over 72 hours. At the conclusion of the CNI taper a loading dose of 2 – 10 mg SRL was given once, followed by a maintenance dose of at least 2 mg SRL daily, adjusted to maintain whole-blood trough levels of 5 – 10 ng/mL. Calculated creatinine clearance and GFR were significantly higher in the SRL arm. Biopsy-proven acute rejection was lower in the SRL arm (14 patients vs. 17 patients; 3 graft losses vs. 6 graft losses). There were no deaths in the SRL arm compared with 5 in the control arm.

CONVERT: Conversion from calcineurin inhibitors to sirolimus maintenance therapy in renal allograft recipients (Schena et al., 2009) evaluated conversion to SRL maintenance 6 – 120 months post-transplant. 830 patients receiving cyclosporine or tacrolimus (along with corticosteroids and either MMF or azathioprine) were randomly assigned to continue CNI (n = 275) or convert from CNI to SRL (n = 555). Patients on the SRL arm received a single loading dose ranging from 12 to 20 mg between 4 to 24 hours after the last dose of CNI. On day 2 of the conversion, they received doses of SRL of 4 – 8 mg daily, adjusted to target whole-blood trough levels of 8 – 20 ng/mL. Upon reaching target SRL levels, MMF/azathioprine could be discontinued per investigator's discretion. GFR at 12 and 24 months was significantly higher in the SRL arm. Rates of biopsy-proven acute rejection and patient survival were similar in the two groups. Urinary protein-to-creatinine ratios increased significantly after SRL conversion. Malignancy rates were significantly lower in the SRL arm.

In summary, the SMART, CONCEPT, STN and CONVERT trials demonstrated similar patient and graft survival in patients who converted to sirolimus. However, patients converting to

sirolimus tended to be at higher risk of edema, hyperlipidemia and new-onset diabetes after transplantation (NODAT). Patients on SRL arms did have lower rates of malignancy. Similar studies have been conducted with everolimus conversion with similar results (Budde et al., 2011; Holdaas et al., 2011; Zeier et al., 2013). It should be noted that in contrast to its use in aplastic anemia or non-myelosuppressive HSCT, immunosuppression in solid organ transplantation is life-long.

2.7 Sirolimus in mouse models of Aplastic Anemia

Dr. Young and his research group have developed a mouse model of immune mediated bone marrow failure (Bloom et al., 2004). (BALB1/cBy x B6) F1 (CByB6F1, $H2^{b/d}$) hybrid mice were conditioned with 5 Gy total body irradiation before infusion of 5×10^6 lymph node cells from H2-disparate C57BL/6 (B6, $H2^{b/b}$) donors. The activated lymphocytes infiltrate the recipient's bone marrow and initiate an immune response that destroys the vast majority of bone marrow cells as *bystanders* (Chen et al., 2004). Within 1-2 weeks of the infusion, recipients developed severe pancytopenia.

Recently this model was used to study immunosuppressive therapies in the treatment of immune mediate bone marrow failure (Feng et al., 2015). Mice were treated EITHER with (i) CsA (50 mg/kg/day x 5 days) starting 1 hour after lymphocyte injection, (ii) sirolimus (2 mg/kg/day x 5 days) starting 1 hour after lymphocyte injection, (iii) sirolimus (2 mg/kg/day x 10 days) starting 3 days after lymphocyte injection OR (iv) sirolimus (2 mg/kg/day x 14 days) starting 1 hour after lymphocyte injection.

All mice receiving only short-term (5 days) CsA or sirolimus died within 30 days of the lymphocyte injection. All mice receiving sirolimus through day 14 survived longer than 100 days until the termination of the experiments.

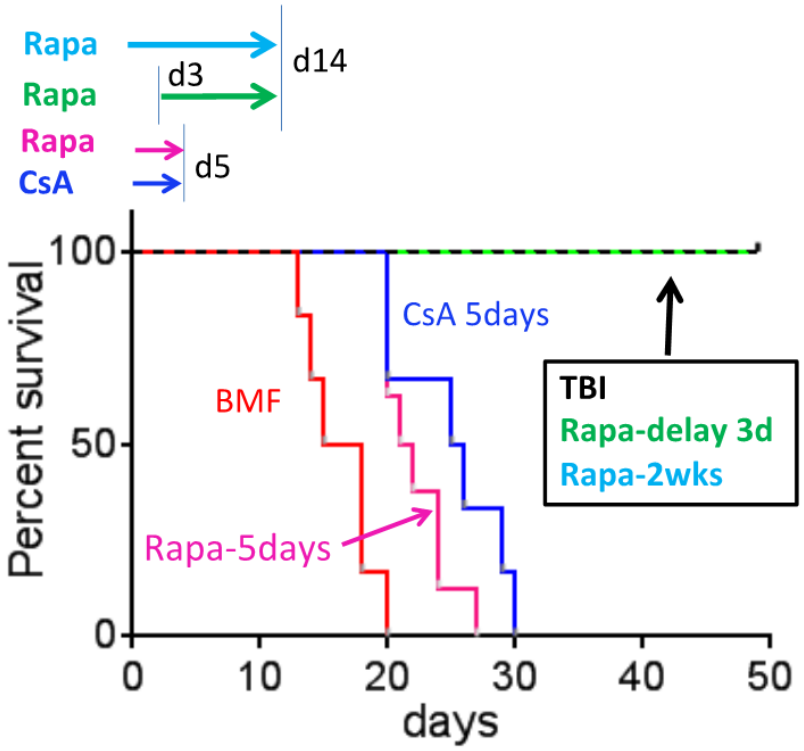


Figure 1. Survival Curves in murine model of immune-mediated bone marrow failure with labeled treatments (Feng et al., 2015).

Cell line counts recovered to a better degree and bone marrow cellularity was higher after sirolimus.

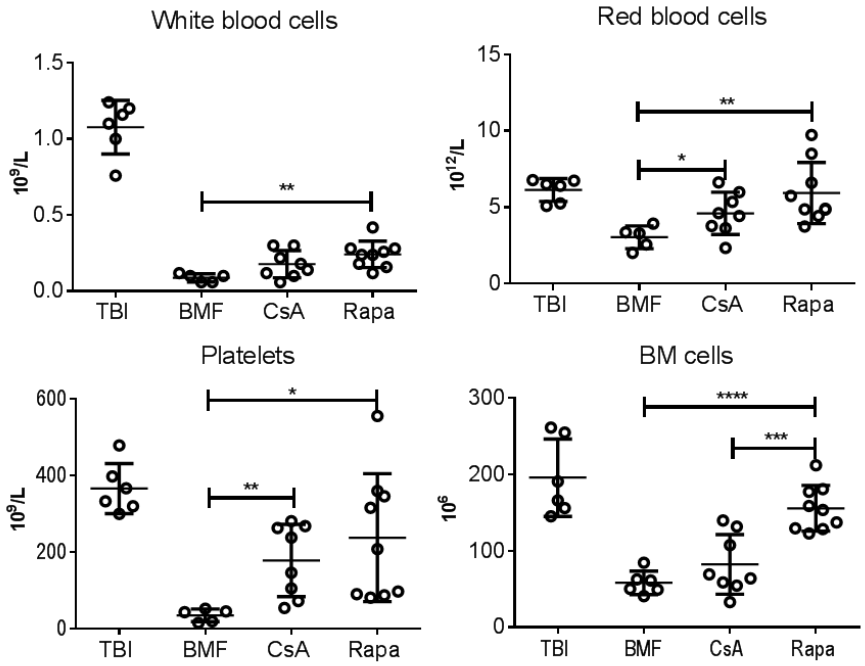


Figure 2. Cell line counts after various treatments in murine model of immune-mediated bone marrow failure (Feng et al., 2015).

Clues to the etiology came from the finding that mice receiving sirolimus had fewer CD8+ T-cells in their bone marrows.

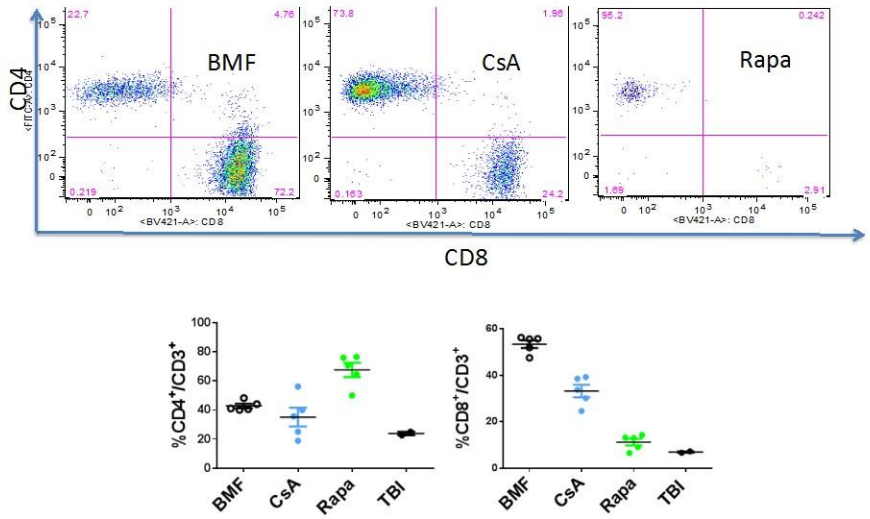


Figure 3. CD8+ bone marrow T-cells after various treatments (BMF = None, CsA = Cyclosporine, Rapa = Sirolimus) in murine model of immune-mediated bone marrow failure (Feng et al., 2015).

Sirolimus treated mice showed decreased T_H1 cytokines and increased T_H2 cytokines.

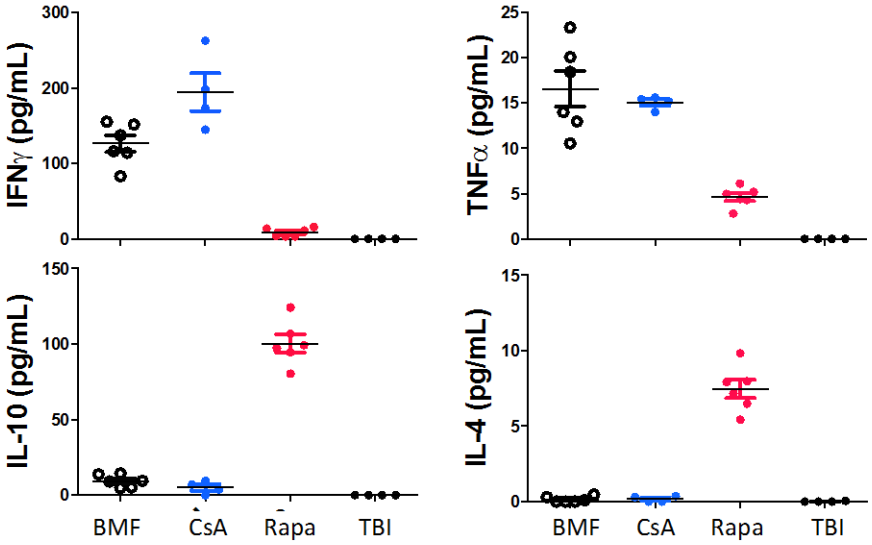


Figure 4. Sirolimus reduced Th1 cytokines, but increased Th2 cytokines in murine model of immune-mediated bone marrow failure (Feng et al., 2015).

Sirolimus-treated mice had expanded regulatory T-cells (Tregs) in their spleens.

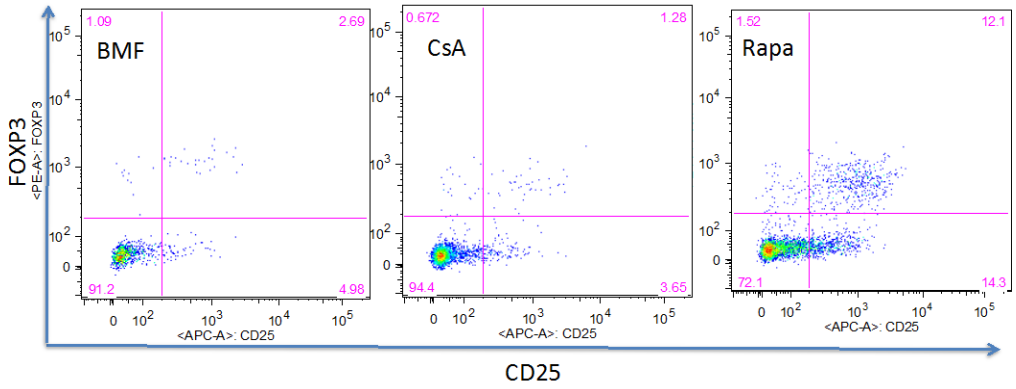


Figure 5. Sirolimus increased counts of splenic Tregs in murine model of immune-mediated bone marrow failure.

These results are consistent with SRL rescuing the recipient mice by inducing tolerance in the donor lymphocytes.

2.8 mTOR Inhibitors in auto-immune diseases

Activation of the mTOR pathway is associated with several rheumatic diseases, including systemic lupus erythematosus, glomerulonephritis/interstitial nephritis, rheumatoid arthritis and systemic sclerosis (Perl, 2015). After the potency of SRL in blocking T-cell activation was discovered, SRL found application in the treatment of SLE. In an MRL/lpr mouse model of lupus, treatment with SRL was found to significantly decrease immunological markers (anti-double-stranded DNA antibody), reduce pathologic features (sizes of spleen and lymph nodes), decrease inflammatory changes (lung, liver, kidney and spleen) and increase overall survival(Warner et al., 1994). A small clinical investigation of 16 patients with clinically active SLE that had been refractory to treatment were randomized to either continuation of current therapy (n = 7) versus continuation of current therapy with addition of SRL (n = 9). Patients in the SRL arm experienced lower British Isles Lupus Assessment Group scores, lower SLE Disease Activity Indices and lower requirement for corticosteroids(Fernandez et al., 2006). A prospective open-label clinical

trial of SRL in 40 patients with SLE has completed in 2015 (NCT00779194); evaluation of results is pending.

Shortly after its adoption in solid organ transplantation, SRL was considered as a potential disease-modifying anti-arthritic drug. In murine models of adjuvant arthritis, SRL inhibited T-cell mediated inflammation and the effects were maintained even after cessation of SRL (Carlson et al., 1993). Over the interim clinical evidence for the use of mTOR inhibitors has been very limited. One clinical study was published reporting on a multi-center, randomized, double blind, placebo-controlled trial of 121 patients with active rheumatoid arthritis (RA) receiving methotrexate (Bruyn et al., 2008). Patients were randomized to continuing MTX either with or without everolimus for 12 weeks. The response rate by American College of Rheumatology criteria of 20% improvement in disease activity (ARC2) was higher in the everolimus group (36.1% versus 16.7%, p = 0.022). Adverse effects were common (GI 52.5%, skin 29.5% and nervous system 21.3%). Interest in this study was limited due to the only moderate benefit but much higher incidence of adverse effects. More recently in a murine model of arthritis, human TNF-transgenic mice, in whom a chronic inflammatory and destructive arthritis develops within 6 weeks after birth were treated with SRL or everolimus and mice were randomized to SRL 10 mg/kg 5 times/week, everolimus 10 mg/kg 5 times/week, placebo 5 times/week (negative control) or infliximab 10 mg/kg 3 times/week (positive control). The study found that mTOR inhibition reduced synovial osteoclast formation and protected against local bone erosions and cartilage loss. Novel biological therapies have revolutionized the treatment of RA for patients. However, about 30% - 40% of patients fail to even achieve a 20% improvement in their disease. mTOR inhibitors may become a useful therapeutic option targeting intra-articular cells that mediate erosive joint destruction (Olsen, 2009; Reynolds et al., 2014).

More recently SRL has been tried in a prospective multi-institutional trial for treatment of relapsed/refractor autoimmune cytopenias (Bride et al., 2016). All 12 children with autoimmune lymphoproliferative syndrome (ALPS) achieved durable complete response after SRL as monotherapy. Eight of 12 children with multilineage cytopenias secondary to common variable immunodeficiency (CVID), Evans syndrome (ES) or systemic lupus erythematosus (SLE) achieved complete response, though the time to response was slower than for the ALPS cohort. Finally, only 2 of 6 children with other auto-immune cytopenias responded. All of the ALPS patients experienced durable responses and chose to remain on SRL beyond the study goal of 6 months; only one ALPS patient chose to discontinue SRL and by the time of publication had not experienced any exacerbation of their underlying cytopenia.

2.9 Scientific and Clinical Justification of the Protocol

Much effort has gone into improving the rate of response of SAA patients to medical therapy. High response rates are achieved with immunosuppressive therapy to decrease or eliminate auto-reactive T-cells in conjunction with growth factors to stimulate proliferation of remaining hematopoietic stem cells (Townsley et al, 2015). Unfortunately, about 30 – 40% of SAA patients, who respond to standard immunosuppressive therapy, eventually relapse. The mechanism for relapse is not understood. One plausible explanation is that auto-reactive $CD8^+$ T-cell clones persist through the immunosuppressive therapy but are functionally suppressed; after withdrawal of the immunosuppressive therapy, the clones expand and become active effectors (Scheinberg et al., 2014). The efficacy of a prolonged CNI taper with regard to decreasing relapse rates was studied in 102 SAA patients, who had been randomized to the control arms of two sequential clinical protocols and who had received the standard immunosuppressive regimen (hATG x 4 days and CsA x 6 months). Sixty-seven of these patients (66%) experienced a hematological response by 6 months of treatment and were subsequently maintained on a prolonged CsA taper for an additional 18 months (Scheinberg et al., 2014). The 5-year rate of relapse rate in this cohort of patients was 33% (95% C.I. 27 – 44%), which did not differ significantly from historical controls in whom CsA was discontinued after 6 months (36%; p = 0.334). Median time

to relapse in the prolonged taper cohort was approximately 2 years, which was about 1 year longer. There were no statistically significant differences between the two cohorts with regard to (i) overall survival, (ii) rate of evolution to a clonal hematopoietic disease or (iii) responsiveness to CsA at relapse.

A role for mTORi in the treatment of patients with SAA has been considered in the past. *Concurrent* treatment of patients with SAA with CNI and mTORi did not result in higher response rates (Scheinberg et al., 2009). Since these clinical trials were completed much has been learned about the complicated role of IL-2 in the activation and homeostasis of the immune system (Boyman and Sprent, 2012). The role of IL-2 in protective immunity was appreciated early on (Malek, 2008; Morgan et al., 1976). IL2 stimulation, is essential for naïve TCR-activated $CD4^+$ T-cells with co-stimulation to differentiate into effector T-cells and for effector $CD4^+$ cells to proliferate. For $CD8^+$ T-cells, IL-2 signaling optimizes the fraction of T-cells that differentiate into effector versus memory T-cells. The critical role of IL-2 in peripheral tolerance became clear only later (Malek and Bayer, 2004). Rather than develop immunodeficiencies, *Il-2*$^{-/-}$ knock-out mice develop lethal auto-immunity (Sadlack et al., 1993). An explanation for this finding came when it was realized that IL-2 is essential for (i) the homeostasis of Tregs (D'Cruz and Klein, 2005; Fontenot et al., 2005; Setoguchi et al., 2005) and (ii) the activation-induced cell death of effector T-cells. IL-2 expression is critical to tolerance.

CNIs interfere with the TCR signaling cascade, resulting in decreased IL-2 expression, inhibition both the de novo generation of Tregs as well as the expansion of existing Tregs (Baan et al., 2005; Gao et al., 2007; Miroux et al., 2009; Wood and Sakaguchi, 2003). For this reason, CNI are likely to be detrimental to the tolerogenicity of the mTORi.

The balance of the roles of IL-2 in protective immunity and peripheral tolerance likely integrates many signals. Experience with mTORi in the transplant setting has taught us that they are not magic bullets that can terminate florid transplant rejection or GvHD. The achievement of stable mixed chimerism in recipients of non-myeloablative hematopoietic stem cell transplants with SRL immunosuppression (Fitzhugh et al., 2013; Hsieh et al., 2014) and the long-term survival of mice treated with mTORi in murine models of bone marrow failure (Feng et al., 2015) support the conjecture that mTORi introduced at the inception of an immune response will induce tolerance. In this understanding of the treatment of SAA, the role of conventional immunosuppressive therapy is to achieve disease remission without necessarily achieving tolerance. The role of eltrombopag is to expand the hematopoietic system to a degree where controlled relapse can be permitted. Finally, the role of mTORi would be to achieve immunological tolerance.

2.10 Rationale for Dose Selection and CNI overlap

Sirolimus will be started at a loading dose of 5 mg PO q4h x 3 doses for patients, followed initially by 5 mg PO daily, adjusted to target a trough serum sirolimus SRL concentration of 10 - 15 ng/mL. This dosing schedule is identical to that already used for non-myeloablative non-myeloablative hematopoietic stem cell transplants (Hsieh et al., 2014); it is also not substantially different from the dosing schedules used for CNI to mTORi conversion in the renal transplant setting (Guba et al., 2012; Lebranchu et al., 2009; Schena et al., 2009; Weir et al., 2011).

CNI was overlapped with mTORi in the SMART trial(Guba et al., 2012). On day 1 of conversion, the CsA dose was reduced by 50%; CsA was discontinued after an overlap of 3 days.

By contrast, the CONCEPT, STN and CONVERT trials (Lebranchu et al., 2009; Schena et al., 2009; Weir et al., 2011) all stopped CNI immediately prior to initiation of SRL. In the non-myeloablative hematopoietic stem cell transplant protocol (Hsieh et al., 2014), SRL is initiated immediately after cyclophosphamide. Exogenous IL-2 can lead to the reversal of anergy, a phenomenon that has been known for decades (Boussiotis et al., 1994), but has only recently

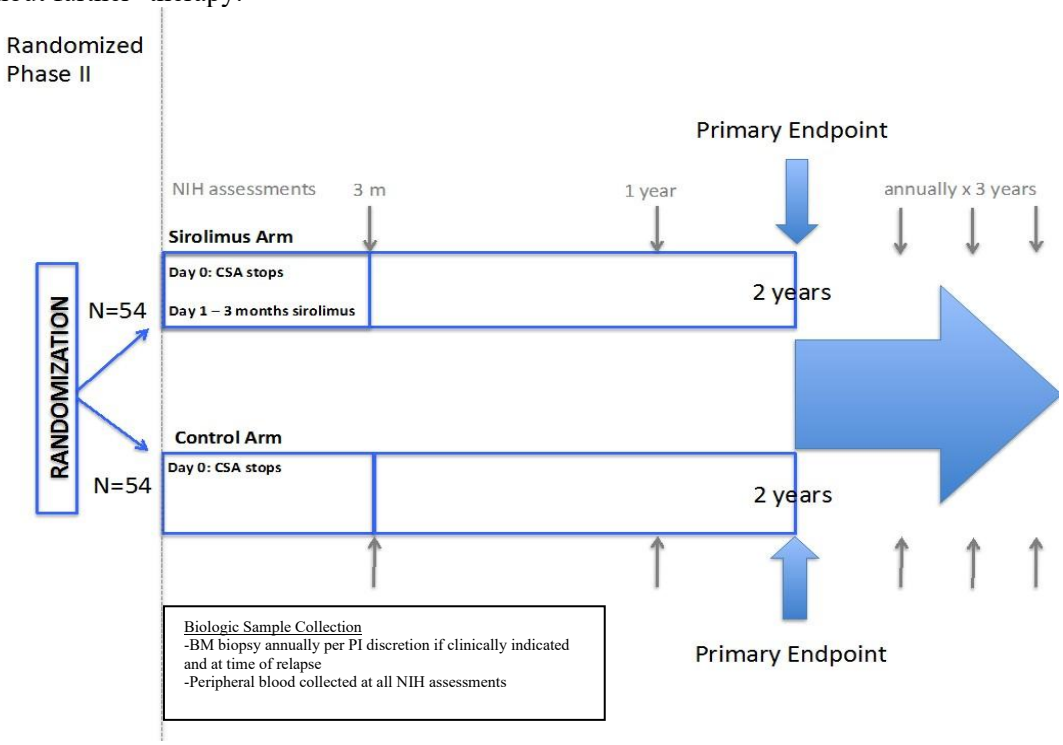
found a molecular explanation (Gao et al., 2012). mTORi appear to block IL-2 induced reversal of anergy (Delgoffe et al., 2011; Powell and Delgoffe, 2010), however the molecular mechanism proposed by Gao et al suggests that this blockade is incomplete. Therefore, we shall suppress IL-2 production by overlapping with CNI.

2.11 Sirolimus use in the pediatric population

Sirolimus has been used in the pediatric setting for more than 15 years. It was employed as a rescue agent in 42 pediatric patients and as primary immunosuppression in 8 pediatric patients (Sindhi et al., 2001) who had received liver (n=26), heart (n=5), intestinal (n=5), liver-intestine (n=9), lung (n=1), bone marrow (n=1), liver-kidney (n=1) and multi-visceral (n=1) transplants. SRL was generally well-tolerated. CSA to mTORi conversion was tried in pediatric renal transplant patients (Ibanez et al., 2005; Vilalta et al., 2003) where results were comparable to the experience in adults. SRL was tried in pediatric liver transplant patients (Markiewicz et al., 2003; Sindhi et al., 2002) where again the adverse event profile was similar to that in adults. As a single agent, sirolimus was recently shown effective and safe in pediatric patients with autoimmune cytopenias associated to autoimmune lymphoproliferative syndrome (Bride et al. Blood 2016).

3.0 Study Design

This is an unblinded, randomized controlled Phase 2 trial where patients with severe aplastic anemia, who responded to IST and currently maintained on CSA, will be randomized to discontinuation of CSA followed by SRL for 3 months (12 weeks), or discontinuation of CSA without further therapy.



Note: Per PI discretion, the 3 Month (12 weeks), 12 months, 36 months, 48 months, and 60 months assessments can be performed at patient's local physician office along with a telehealth visit with a provider of the research team (PI/AI)

Figure 6. Diagram of trial design; 54 evaluable patients will be randomized to each arm of the trial. Relapsed patients on both arms will exit the study.

3.1 CSA to Sirolimus conversion

Current CSA treatment will be stopped on day 0 for both arms. Sirolimus will be started on day 1 for subjects in the Sirolimus arm. A baseline CBC will be obtained within one week of

randomization, or on Day 0. Thereafter, CBC will be monitored on a 1-2 weeks basis for subjects in both arms. In the event of relapse (see definition of outcomes Section 6.7), the patient may return to the NIH CRC for evaluation to include bone marrow biopsy and aspirate to exclude clonal evolution to MDS. Assessment will include (i) flow cytometry phenotyping of peripheral blood mononuclear cells for regulatory T-cells (Tregs, e.g. $CD4^{+}$ $CD25^{high}$ $FOXP3^{+}$) and regulatory dendritic cells (DCregs) and (ii) proteomics assay (research labs).

4.0 Eligibility Assessment and Enrollment

All subjects age 2 and older with a history of SAA who received IST with a lymphodepletion-based regimen, achieved a hematologic response, and are currently maintained on CSA will be considered for enrollment.

Eligibility will be determined either on another active Hematology Branch protocol or on the NHLBI screening protocol (97-H-0041). The time between eligibility determination and signing consent to participate on this protocol may not exceed 90 days.

4.1 Inclusion Criteria

1. Age ≥2 years old
2. Weight > 12 kg
3. Previous diagnosis of SAA by bone marrow biopsy and cytogenetics, treated with lymphodepleting therapy (ATG, cyclophosphamide or alemtuzumab) that included cyclosporine. The lymphodepleting therapy must have been administered at least 12 months prior.
4. Continuous treatment with cyclosporine for the previous 6 months (excluding minor dose delays not exceeding more than 30 days).
5. Evidence of a hematologic response to an lymphodepletion-based regimen as evidence of at least two of the following:
   - Absolute neutrophil count ≥ 500/uL
   - Platelet count ≥ 20,000/uL (without transfusion support)
   - Absolute reticulocyte count ≥ 60,000/uL
     (or hemoglobin 10 gm/dL without transfusion support)

4.2 Exclusion Criteria

1. Evidence of relapse of aplastic anemia due to cyclosporine withdrawal during the previous 6 months
2. Prior use of sirolimus or other mTOR inhibitor within 12 weeks of study entry
3. Myelodysplastic syndrome or acute myeloid leukemia, according to WHO diagnostic criteria (if baseline BM consistent with MDS after enrollment, patients will be considered ineligible and immediately exit the study, and the subject can be replaced with another subject)
4. Patients that are on CYP3A4 inhibitors and cannot replace these medications with other equivalent medications for the period of study: protease inhibitors (ritonavir, indinavir, nelfinavir, saquinavir), some macrolide antibiotics (clarithromycin, telithromycin, erythromycin), azole anti-fungals (fluconazole, itraconazole, ketoconazole), metroclopramide, felodipine, nifedipine, carbamazepine, phenobarbital, grapefruit juice and St. John's Wort.
5. Anaphylactic or hypersensitivity reaction to sirolimus
6. Patients with infections not adequately responding to appropriate therapy as evidenced by persistence of a clear source of infection that, in the view of the investigator, would preclude safe treatment with sirolimus.
7. Current pregnancy, or unwillingness to take oral contraceptives or use the barrier methods of birth control or practice abstinence to refrain from pregnancy if of childbearing potential during the course of the study.

8. Lactating women, due to the potentially harmful effects on the nursing child.
9. Patients who have received live vaccines within the past 30 days
10. Patients with cancer who are actively receiving chemotherapeutic treatment or who take drugs with hematological effects such as thrombopoietin receptor agonists (such as eltrombopag), granulocyte-colony stimulating factor or erythroid stimulating agents.
11. Moribund status such that death within 7 to 10 days is likely. Comorbidities of such severity that in the view of the Investigator it would likely preclude the patient's ability to tolerate sirolimus.
12. Inability to understand the investigational nature of the study or to give informed consent or without a legally authorized representative or surrogate that can provide informed consent.

Patient Registration and Treatment Randomization

Patients will be evaluated in the hematology clinic during which time the eligibility checklist will be completed. After confirmation of eligibility and obtaining written consent, the principal investigator or the research nurse will notify the pharmacy of the subject's enrollment and the pharmacy will randomize the subject to therapy. Assignment of treatment will be done using a block randomization scheme with the assignment probability remaining fixed over the course of the trial. Construction of the randomization schedule is done by pharmacy using a table of random numbers, taken from Documenta Geigy, Scientific Tablets, seventh edition.

We will use the block randomization process described in Friedman, Furberg and DeMets (2010, Chapter 6). Each block will have 6 subjects with 3 randomized to the Sirolimus arm and 3 to the control arm. This will lead to an overall 18 blocks. Within each block, the assignment of the patients to either the Sirolimus arm or the control arm will be decided by a table of random numbers.

4.3 Screening Evaluation

Patients will be evaluated with complete medical history, physical exam and the following tests at the NIH or with outside referring hematologist:

- Complete blood count with differential
- Reticulocyte count
- Direct antiglobulin test (at the PI's discretion)
- Serum or urine beta-hCG for female patients of childbearing age and anatomic ability
- Confirmation of the diagnosis of aplastic anemia by NIH records or outside records demonstrating a bone marrow report(s) and blood counts consistent with aplastic anemia prior to treatment with IST.
- Acute Care Panel (Na, K, Cl, CO_2, Creatinine, Glucose, and Urea Nitrogen), Mineral Panel (Phosphorus, Magnesium, Albumin, and Calcium), Hepatic Panel (Alk Phosphatase, ALT, AST, and Total Bilirubin). Direct bilirubin will be requested when indicated based on total bilirubin.

Laboratory results from other NIH Intramural IRB approved protocols that subjects may have participated in or from outside referring hematologist within 6 months of enrollment in this study may be used as part of the pre-study evaluation for prescreening labs for this study.

4.4 Baseline Studies

The following studies will be conducted at the NIH or test results from outside referring hematologist prior to but within 90 days of Day 0 and group randomization may be used with exceptions noted*. These studies can also be considered baseline if done under other NIH protocols (see Section 4.0) or from outside referring hematologist within 90 days.

If an interested participant is unable to come to the CC for the initial baseline evaluation, remote enrollment may be considered if all the below listed tests/procedures are done with the referring hematologist. A telehealth visit will be conducted to discuss the protocol in detail, perform a history and limited physical examination, and review the availability of all required baseline testing prior to consenting and randomizing the subject. Certain labs indicated by ** can be performed within 48 hours of randomization.

- Hematological profile: CBC with differential, and reticulocyte count
- Iron studies (serum iron, transferrin and ferritin)
- *Bone marrow aspiration and core biopsy with chromosomal analysis via standard cytogenetic techniques (can be considered baseline if conducted at the NIH after the enrollment no longer than Day 14 of group randomization)
- Flow cytometry of the peripheral blood to quantitate GPI-negative cells
- Peripheral blood lymphocyte phenotype via flow cytometry
- Acute Care Panel (Na, K, Cl, CO_2, Creatinine, Glucose, and Urea Nitrogen), Mineral Panel (Phosphorus, Magnesium, Albumin, and Calcium), Hepatic Panel (Alk Phosphatase, ALT, AST, Total Bilirubin, and Direct Bilirubin), and other panel (Uric Acid, Lactate Dehydrogenase, Creatine Kinase))
- Fasting lipid panel; including total cholesterol, HDL, LDL and triglycerides**
- Fasting glucose**
- Hemoglobin A1C**
- Urinalysis**
- Urinary protein, creatinine and albumin**
- Serum or urine beta-hCG for female patients of childbearing age and anatomic ability
- Viral serologies for CMV, EBV, hepatitis A, B, C, HIV 1/2, HSV**
- CMV and EBV PCR**
- Type and Antibody Screen

5.0 Treatment Plan

5.1 Cyclosporine (CsA)

Cyclosporine will be discontinued in all subjects randomized to both arms of the study on day 0. For subjects enrolled remotely, day 0 for the sirolimus arm may not be the day of signing the consent but it will be the day the subject confirms receipt of sirolimus and the team instructs to stop cyclosporine and initiate sirolimus.

5.2 Sirolimus Arm Initial dosing

Patients will be started on day 1 with one loading dose of 6mg on day 1, followed by 2mg/day in adults, and in children less than 40 kg the loading dose will be 3 mg/m^2 followed by 1 mg/m^2/day. The initial dosage in patients ≥ 13 years who weigh less than 40 kg should be adjusted, based on body surface area, to 1 mg/m^2/day. Sirolimus will be taken orally once a day for 3 months (12 weeks). Dose will be adjusted to maintain a sirolimus trough level in the blood between 5- 15 ng/mL. The sirolimus trough levels will be assessed prior to the next scheduled dose. Increases in sirolimus blood concentrations occur when the drug is administered at the same time as CSA, therefore the initial dose of sirolimus on day 1, should be administered at least 4 hours after the last dose of cyclosporine.

For subjects enrolled remotely – Cyclosporine will be continued until sirolimus is received by the subject. Once subject receives the drug, the team will instruct to discontinue cyclosporine and initiate sirolimus at least 4 hours after the last dose of cyclosporine.

Dose-adjustments and delays

Elderly patients (>65 years) or patients with hepatic impairment or significant comorbidities may

have the loading dose and/or initial dose of SRL reduced at the primary investigator's discretion. The loading dose of sirolimus and the maintenance dose of sirolimus may also be reduced in patients with mild to severe hepatic impairment. In subjects receiving concomitant medications known to interact with sirolimus, dosage adjustments may be implemented at the primary investigator's discretion. Dosage adjustment is not required in patients with renal impairment. Dose adjustments to maintain a goal SRL trough level will not be performed if, at the discretion of the primary investigator, intolerable toxicity attributable to SRL is observed.

Dose delays of SRL will be permitted for maintaining goal trough levels, or if minor (less than 3 days). Sirolimus may be held at the discretion of the PI for serious adverse events, but these will be reported in the SAE reporting.

Drug interactions
Patients will be advised not to ingest grapefruit juice or St. John's Wort due to the interference with the metabolism of sirolimus through the cytochrome p-450 complex. Patients will also be advised to avoid concomitant use with strong CYP3A4/P-gp inducers or strong CYP3A4/P-gp inhibitors that decrease or increase sirolimus concentrations while sirolimus is administered.

- *Drugs that could **increase sirolimus** blood concentrations:* Bromocriptione, cimetidine, cisapride, clotrimazole, danazol, diltiazem, fluconazole, protease inhibitors (e.g., HIV and hepatitis C that include drugs such as ritonavir, indinavir, boceprevir, and telaprevir), metoclopramide, nicardipine, troleandomycin, verapamil

- *Drugs and other agents that could **decrease sirolimus** concentrations:* Carbamazepine, phenobarbital, phenytoin, rifapentine, St. John's Wort (*Hypericum perforatum*)

- *Drugs with concentrations that could increase when given with sirolimus:* Verapamil

Vaccinations
Immunosuppressants like SRL may affect response to vaccination. Therefore, during treatment with SRL, vaccination may be less effective and patients will be advised to avoid live vaccinations during SRL administration.

5.3 Control Arm
Subjects in the control arm will have cyclosporine discontinued on the day of randomization, day 0. Subjects will not receive any treatment or placebo under this protocol.

6.0 Clinical monitoring

6.1 On study monitoring - Day 0 (+ 48hours for certain labs marked by **)
On treatment monitoring will consist of (does not need to be repeated if performed in previous 2 weeks):

- Pregnancy test (urine or serum hCG in women of child bearing potential).
- History and physical examination.
- Hematological profile: CBC with differential, reticulocyte count and platelet count.
- Peripheral blood lymphocyte phenotype via flow cytometry
- Acute Care Panel (Na, K, Cl, CO_2, Creatinine, Glucose, and Urea Nitrogen), Mineral Panel (Phosphorus, Magnesium, Albumin, and Calcium), Hepatic Panel (Alk Phosphatase, ALT, AST, and Total Bilirubin).
- Urinary protein, creatinine and albumin**
- Fasting lipid panel; including total cholesterol, HDL, LDL and triglycerides**
- Hemoglobin A1C**
- Fasting glucose**

- Research biological sampling of peripheral blood

6.2 On study monitoring – Day 1 to 5 years

- Hematological profile: CBC with differential and reticulocyte count (if available) at least every 2 weeks (+/- 4 days) for 3 months (12 weeks), thereafter at least once every 4 to 8 weeks (+/- 14 days), at PI discretion, until 24 months, then every 12 weeks – 24 weeks (+/- 28 days) until 5 years.
- Acute Care Panel (Na, K, Cl, CO_2, Creatinine, Glucose, and Urea Nitrogen), Mineral Panel (Phosphorus, Magnesium, Albumin, and Calcium), Hepatic Panel (Alk Phosphatase, ALT, AST, Total Bilirubin) at least every 4 weeks (+/- 7 days) while receiving sirolimus or until landmark 3-month visit.
- Fasting lipid panel; including total cholesterol, HDL, LDL and triglycerides at least every 4 weeks (+/- 7) days while receiving sirolimus or until 3-month landmark visit.
- Whole blood sirolimus trough level at least every 7 days (+/- 3 days) for the first month, then at least every 14 days (+/- 4 days) while receiving sirolimus or until 3-month landmark visit. (Sirolimus arm only)

6.3 Landmark 3-month (12 weeks) follow up visit (Both arms):

Per PI discretion, the 3-month/12 weeks (+/- 7 days) follow up visit can be performed at the NIH Clinical Center or at the local physician's office.

Monitoring includes:

- Pregnancy test (urine or serum hCG in women of child bearing potential).
- History and physical examination.
- Hematological profile: CBC with differential, reticulocyte count and platelet count.
- Peripheral blood lymphocyte phenotype via flow cytometry (done at the NIH per PI discretion – optional)
- Acute Care Panel (Na, K, Cl, CO_2, Creatinine, Glucose, and Urea Nitrogen), Mineral Panel (Phosphorus, Magnesium, Albumin, and Calcium), Hepatic Panel (Alk Phosphatase, ALT, AST, and Total Bilirubin), and other panel (Uric Acid, Lactate Dehydrogenase, Creatine Kinase, Cystatin C).
- Urinalysis
- Type and Antibody Screen (done at the NIH per PI discretion - optional)
- Urinary protein, creatinine and albumin (at the PI's discretion - optional).
- Fasting lipid panel; including total cholesterol, HDL, LDL and triglycerides (done at the NIH per PI discretion for observation arm – optional)
- Hemoglobin A1C (at the PI's discretion - optional)
- Fasting glucose (at the PI's discretion - optional)
- Research biological sampling of peripheral blood (done at the NIH per PI discretion - optional)
- Flow cytometry of the peripheral blood to quantitate GPI-negative cells (done at the NIH per PI discretion - optional)

6.4 Yearly landmark visits (12, 24, 48 and 60 months)

After the 3 month visit, patients enrolled on the study must be evaluated at the Clinical Center at 24 months (+/-60 days) except for the situations listed below*. Landmark 12 months (+/- 30 days), 36 months (+/- 90 days), 48 months (+/- 90 days), and 60 months (+/-90 days) may be conducted at the local physician's office along with a telehealth visit with a provider of the research team (PI/AI). Per PI discretion, participants may be evaluated more frequently at the NIH if medically indicated based on disease status. The following monitoring will be conducted at each clinic visit at the CC or at the

local physician's office:

*During public health emergencies or special circumstances beyond the subjects control, if a subject is unable to present to the CC, the research team will coordinate with the home physician a visit and request from the home physician the following listed tests. In addition, a telehealth visit will be conducted by a provider of the research team (PI/AI) to replace the CC visit. For international subjects where the resources don't allow the listed tests/procedures below, we will conduct a phone visit or other NIH approved platform and allow the subject an additional 60 days to present to the CC for complete evaluation.

- Hematological profile: CBC with differential, reticulocyte count and platelet count
- Bone marrow aspiration and core biopsy with chromosomal analysis via standard cytogenetic techniques per PI discretion if clinically indicated
- Acute Care Panel (Na, K, Cl, CO_2, Creatinine, Glucose, and Urea Nitrogen), Mineral Panel (Phosphorus, Magnesium, Albumin, and Calcium), Hepatic Panel (Alk Phosphatase, ALT, AST, Total Bilirubin, and Direct Bilirubin), and other panel (Uric Acid, Lactate Dehydrogenase, Creatine Kinase)
- Urinalysis
- Type and Antibody Screen
- Flow cytometry of the peripheral blood to quantitate GPI-negative cells
- Research biological sampling of peripheral blood
- The following will be collected at the PI's discretion:
    - Urinary protein, creatinine and albumin
    - Fasting serum glucose
- The following will be collected only at 12 months unless clinically indicated:
    - Fasting lipid panel

6.4 Relapse

In the event that relapse criteria are met, research biological sampling of peripheral blood may be obtained at the NIH or mailed to the NIH. Subjects may be asked to return to the NIH for evaluation that includes a bone marrow biopsy to evaluate for progression to MDS/AML per PI discretion if clinically indicated.

6.5 Long-term follow up outpatient

Patients on the study will be seen by their home physician as clinically indicated. The Hematology Branch investigators and home physicians will remain in communication. Home physicians will monitor CBC and other clinical parameters as clinically indicated.

6.6 Criteria for Relapse

For the purpose of this protocol, we shall define patients as having ***relapsed***, if after enrollment, EITHER:

(1) Their cell line counts decline to a degree where they again meet Camitta criteria for severe aplastic anemia (determined from at least 2 CBCs done within 14 days)

OR

(2) The primary investigator observes a decline in blood counts, not easily explained by another clinical process exhibiting a temporary impact on blood counts, and meets the following criteria:
    a. A rapid drop in one or more blood cell lineages, or a progressive decline in one or more blood cell lineages on at least two consecutive blood draws as determined by the primary investigator.
    b. Two blinded investigators, John Tisdale and Phillip Scheinberg, will review the

trend in blood counts and clinical scenario and must also agree with the relapse assessment.

6.7 Research Studies

Intended use: During this study, blood (at baseline, 3 months (12 weeks) and annually thereafter) and bone marrow aspirate (at baseline, at 3 months (12 weeks) and at Year 2, 3, 4 & 5), may be obtained for the research studies listed below. Baseline samples may be obtained under protocol 04-H-0012. These studies are not used in assessing the primary endpoint but are undertaken as descriptive or exploratory ancillary studies.

Assessment of cytokines and lymphocyte phenotype

We may assay pre- and post-treatment changes in peripheral and bone marrow lymphocyte phenotypes via flow cytometry. Requisite labs will be drawn on D1, 3 mo, and subsequent annual visits.

Assessment of cytokines

We may assay pre- and post-treatment circulating cytokines (IL-1β, IFN-γ, IL-17, IL-6, IL-10, etc.) in the serum. Requisite labs may be drawn on D1, 3 mo, and subsequent annual visits.

Assessment of T-cell receptor repertoire

We may assess pre- and post-treatment T-cell receptor (TCR) repertoire via single cell RNA-seq for high-resolution quantitative analyses of immunological clonality as well as identification of corresponding T-cell subsets. Requisite labs may be drawn on D1, 3 mo, 6 mo and subsequent annual visits.

Assessment of thrombopoietin levels

Thrombopoietin (TPO) has a pleiotropic role in hematopoiesis. Its cognate receptor, c-mpl, is expressed and functional on primitive hematopoietic stem and progenitor cells. TPO levels may be assessed on D1, 3 mo, 6 mo and subsequent annual visits.

7.0 BIOSTATISTICAL CONSIDERATIONS

7.1 Primary Endpoint

The primary endpoint is the rate of relapse in both arms at 24 months (see Section 6.7 for relapse definition). The primary end point of 24 months was chosen as per the study of Scheinberg et al (Scheinberg et al., 2009) the majority of relapses were noted to occur within this time frame.

- Subjects who drop out of the study, for events such as those listed in Section 7.7, death or in rare cases are lost to follow-up, prior to 24 months will be considered as "relapse" for the primary endpoint variable according to the intent-to-treat principle. Subjects who are found ineligible after enrollment due to the baseline bone marrow evaluation identifying a diagnosis alternative to aplastic anemia such as MDS or AML, will not be counted in the statistical analysis and can be replaced with another subject. These subjects will be mentioned in a CONSORT diagram.

7.2 Secondary Endpoints

- Safety and tolerability of sirolimus
- Time to relapse
- Relapse rates prior to 24 months, and after 24 months
- Effects in peripheral blood cell counts and bone marrow cells
- Clonal evolution to myelodysplasia by morphology or new cytogenetic abnormalities
- Development of acute myeloid leukemia

- Clonal expansion of PNH
- Survival at 5 years

Subjects will be followed up to 60 months so that long-term disease-free and overall survival can be estimated. Relapse and toxicity comparisons will also be made with the results obtained from prior NHLBI studies: the h-ATG/CsA protocol (90-H-0146); the h-ATG/CsA vs. Cytoxan/CsA protocol (97-H-0117); the h-ATG/CsA/MMF protocol (00-H-0032); the h-ATG/CsA/Rapamune protocol (03-H-0193); the 3-arm randomized study (06-H-0034); the r-ATG vs. alemtuzumab (03-H-0249) trials and the eltrombopag protocol (12-H-0150).

7.3 Sample size

The study will enroll n=54 evaluable patients per arm, so that the total sample size is N=108 (54 ×2) patients for both the Sirolimus arm and the control arm combined.

To account for the possibility of subjects who are found ineligible after enrollment due to the baseline bone marrow evaluation identifying an alternative diagnosis to aplastic anemia such as MDS or AML, up to 10 additional subjects may be enrolled to replace those subjects that are non-evaluable for the study's primary endpoint. We note that between 1.7% to 15% of patients with severe aplastic anemia treated with IST develop myelodysplastic syndrome. Thus, we request IRB approval to enroll up to 118 subjects.

7.4 Statistical Design

The proposed sample size of 54 patients per arm is calculated based on the following statistical design. Let p0 be the 24-month relapse rate for the control arm, and p1 be the 24-month relapse rate for the Sirolimus arm (relapse here includes death or loss of follow-up at 24 months). Our statistical design is based on testing the following null hypothesis H0: $p0 = p1$ versus the two-sided alternative H1: $p0 \neq p1$ at 0.05 significance level and 80% power.

Our experience based on patients who were treated in Protocol #12-H-0150 suggests that the relapse rate of this patient population in the control arm could be as high as 45%. To demonstrate a meaningful clinical reduction of the relapse rate for the Sirolimus arm, we would like the relapse rate for the Sirolimus arm to be as low as 20% to show efficacy of the Sirolimus therapy. Using the assumption that p0=45% and p1=20%, the two-sided Binomial test for proportions at 0.05 significance level and 80% power requires n=54 patients per arm without continuity correction. Numerical results are computed using the "power.prop.test" command of R or TIBCO Spotfire S+ statistical packages.

We expect to enroll approximately 12 patients per year per arm (24 patients per year for both arms). It will take about 4.5 to 5 years to enroll 54 patients per arm. Approximately 20% of the 54 patients (i.e., 11 patients) will complete their 24-month follow-up at 3 years. Approximately 50% of the 54 patients (i.e., 27 patients) will complete their 24-month follow-up at approximately 4.5 years. We expect to have all 108 patients (54 per arm) randomized and started the treatments between 4.5 to 5 years. Based on the above enrollment projection, we plan to use the Lan-DeMets alpha spending approach (DeMets and Lan, 1993; Proschan, Lan and Wittes, 2006) to monitor the efficacy and futility of the Sirolimus treatment at the "information time" t=0.2, 0.5 and 1.0 (that is, the two interim looks are at time points when 11 and 27 patients per arm, respectively, completed their 24-month follow-up).

Among the three most popular spending functions, namely the O'Brien-Fleming –like spending function, the Linear spending function and the Pocok-like spending function (Proschan, Lan and Wittes, 2006, page 87), the linear spending function appears to be most appropriate for our study. The O'Brien-Fleming-like spending function gives a much too wide stopping boundary at the first interim look (i.e., t=0.2) with z =4.877, which is not satisfied except for the most extreme

case. The Pocok-like spending function, on the other hand, leads to a stopping boundary with z =2.438 at the first interim look (t=0.20), which does not seem realistic because it allows easy early stopping when the sample size is 11 patients per arm. Therefore, we establish the interim analysis stopping rule based on the linear spending function with symmetric lower and upper bounds at 0.05 overall alpha. The following table gives the lower and upper stopping boundaries at information time t=0.2, 0.5 and 1.0, which are computed using "LD" software provided by Reboussin, DeMets, Kim and Lan (1996); see Proschan, Lan and Wittes (2006, page 87).

Lan-DeMets bounds for a given spending function

n = 3
Overall alpha: 0.05

Type: Two-Sided Symmetric Bounds
Lower alpha: 0.025
Upper alpha: 0.025
Spending function: Linear

Boundaries:

	Time	Lower	Upper
1	0.2	-2.576	2.576
2	0.5	-2.377	2.377
3	1.0	-2.141	2.141

The 2.141 critical value at the final look corresponds to a two-sided nominal p-value = 0.032.

We will inform the DSMB about the test statistic at each of the interim look, and consider stopping the trial for efficacy (or futility) when the above stopping boundaries have been crossed. We will re-compute the stopping boundaries if the DSMB decides to take interim looks at time points other than t=0.2, 0.5 and 1.0.

Patient safety will be monitored by a separate statistical stopping rule for safety specified in Section 7.5. Stopping of the study due to safety reasons will be based on the DSMB and IRB recommendations.

7.5 Statistical methods

The planned analyses will be based on the intention to treat principle and will include descriptive statistics on the proportions of relapsers (i.e. % subjects who relapse in each arm), cumulative incidence of relapse with death considered to be a competing risk, and survival analyses on time to relapse and overall survival. The relapse probabilities will be estimated using the sample proportions and their inferences including confidence intervals and hypotheses testing will be evaluated using Binomial distributions and their appropriate normal approximations. The distributions of overall survival and time to relapse will be analyzed using appropriate tools in survival analysis, such as Kaplan-Meier estimates, Cox Proportional Hazard Regression, Frailty models and other generalized hazard regression models taking consideration of random censoring. Graphical tools will be used to display the appropriate estimates (i.e. estimated proportions, distributions of cumulative incidences and Kaplan-Meier curves) and their corresponding 95% confidence intervals. Methods based on multiple regression, analysis of variance and logistic regression will also be employed for secondary endpoint analysis and exploratory analysis if deemed appropriate

7.6 Stopping Rules

The study will be monitored to ensure that the occurrence of a specified set of treatment related serious adverse events (TRSAEs) within 2 years of treatment in the trial does not substantially exceed an anticipated rate. The following TRSAEs will be monitored for early stopping of the study:

- Death considered to be probably or definitely related to SRL.
- Any grade IV toxicity considered to be probably or definitely related to SRL.

The study will be monitored using the stopping rules as outlined below for early stopping if the number of subjects in the study who have developed one or more of the above specified TRSAEs is over a pre-specified threshold value. TRSAEs are those attributed as definitely or probably related to sirolimus. Dr. John Tisdale will serve as the independent monitor who reviews the attribution of TRSAEs. A list of TRSAEs related to death or toxicity of SRL will be provided to the IRB.

From our experience with the patients treated in Protocol 12-H-0150, we anticipate the rate of developing at least one of the above specified TRSAEs for this patient population to be 20% or less. We will monitor the TRSAEs for each of the two arms separately. If the numbers of observed TRSAEs in an arm exceed the stopping boundary, the DSMB will be informed, and the possibility of stopping patient enrollment for this arm or the study will be seriously considered. Following Geller et al. (2004, "Design of Early Trials in Stem Cell Transplantation: A Hybrid Frequentist-Bayesian Approach"), our stopping rule is determined by a Bayesian approach. The stopping boundary for the study is reached if the Bayesian posterior probability that the true probability of developing one or more of the above specified TRSAEs exceeds this benchmark rate of 20% is at least 90%. We take our prior distribution to be a beta distribution with the sum of the two beta parameters to be 6, i.e. the parameters of the beta prior distribution are 1.2 and 4.8. Since we have seen in the past that the first few subjects to be accrued are possibly sicker than the rest of the subjects in the sample, we will start safety monitoring the study when 3 or more subjects have developed specified TRSAEs. The following tables summarize the threshold numbers for stopping patient enrollment in corresponding arm:

Number of SAA subjects enrolled	Stop if the number of SAA subjects who develop any of the specified TRSAEs reaches:
≤ 5	3
≤ 8	4
≤ 12	5
≤ 16	6
≤ 20	7
≤ 24	8
≤ 28	9
≤ 32	10
≤ 36	11
≤ 40	12
≤ 45	13
≤ 49	14
≤ 54	15

We performed a simulation study to evaluate the statistical properties of the stopping rule.

In each simulation run, we generated a study with 54 independent Bernoulli trials, each had a probability p for having the above TRSAE and q=1-p for not having such TRSAE and compared the TRSAE outcomes with the above stopping boundary to determine whether the study was stopped. We repeated the simulation 100,000 times and computed the proportion of stopped studies (i.e. "number of stopped studies"/100,000), which were stopped using the above stopping rule. The following table summarizes the proportions of stopped studies under a number of scenarios for p:

Probability of "Monitored" TRSAE= p	5%	10%	15%	20%	25%	35%
Proportion of stopped studies	0.15%	1.64%	7.91%	25.59%	53.55%	93.80%
Average number of Tx naive SAA subjects	53.93	53.26	50.90	45.25	36.52	19.14
Average number of Tx naive SAA with a specified TRSAE	2.70	5.32	7.63	9.06	9.13	6.69

These results suggest that the above stopping rules have a low probability of stopping a study when the proportion of the corresponding specified TRSAE is below the benchmark value of 20%, and the probability of stopping a study is high when the true proportion of the above specified TRSAE exceeds this benchmark value. Based on these results, we believe that our Bayesian stopping rules have satisfactory statistical properties and is useful for guiding the safety monitoring of this study.

7.7 Off study/off treatment criteria

All subjects will go off study after the 5-year NIH landmark visit.

Subject choice: Subjects may be removed from study at their request. The risks of withdrawing will be discussed, as will alternative treatment options. Those subjects who choose to withdraw will be strongly encouraged to continue to have labs monitored until he/she initiates alternative SAA therapy or through the 6 month off study medication to assess for late occurring adverse events.

Principal investigator decision: Should any of the following adverse events occur, study drug administration will be discontinued and the subject will be followed until resolution of the event and labs are monitored until he/she initiates alternative SAA therapy or through 6 months off study drug time point.

- Any grade IV adverse event considered to be probably or definitely related to SRL
- Life threatening acute hypersensitivity reactions

Should any of the following events occur, study drug administration will be discontinued and the subject will be taken off study.

- Pregnancy or unwillingness to refrain from pregnancy (sirolimus arm only)
- Patients who require chemotherapeutic treatment or require drugs with hematological effects
- Relapse criteria are met
- Evidence myelodysplastic syndrome or AML according to WHO criteria at any time

while on study, or in subjects who were not initially excluded because the baseline bone marrow evaluation was pending at the time of enrollment.

- Any grade IV adverse event considered to be probably or definitely related to SRL (subject will be taken off study upon resolution of the event)

Once off study, subjects will be referred back to his or her referring physician or consented to the Hematology Branch evaluation and treatment protocol (94-H-0010) for consideration for standard therapy or evaluation for eligibility for another Branch protocol, depending on what is considered to be in the best interest of the subject.

Lost to Follow up: Research team was unable to contact the subject on at least three occasions.

Non Compliance: If subject does not comply with medication and/or visit schedule or protocol restrictions

Death: Death of Subject

8.0 Data and safety monitoring

8.1 Safety Monitoring

Principal Investigator: Accrual, efficacy and safety data will be monitored by the Principal Investigator, Bhavisha Patel, M.D.

NIH Intramural IRB: Prior to implementation of this study, the protocol and the proposed patient consent and assent forms will be reviewed and approved by the properly constituted Institutional Review Board (IRB) operating according to Title 45 CFR 46. This committee will also approve all amendments to the protocol or informed consent, and conduct continuing annual review so long as the protocol is open to accrual, follow up of subjects or data analysis.

NHLBI DSMB: The NHLBI Data Safety and Monitoring Board will review the protocol at 6 to 12 month intervals. A progress report will be forwarded to the DSMB at these times and their recommendations will be expeditiously implemented. The DSMB may recommend early termination of the study for considerations of safety and efficacy.

8.2 Assessment of Safety

Definitions

Please refer to Policy 801 for current Definitions.

8.3 Adverse Events (AEs) Characterization

The AEs will be attributed (unrelated, unlikely, possibly, probably or definitely related) to study medication and/or disease. The AEs will be graded by severity utilizing CTCAE version 4.0.

An abnormal laboratory value will be considered an AE if the laboratory abnormality is characterized by any of the following:

- Results in discontinuation from the study
- Is associated with clinical signs or symptoms
- Requires treatment or any other therapeutic intervention
- Is associated with death or another serious adverse event, including hospitalization
- Is judged by the Investigator to be of significant clinical impact
- If any abnormal laboratory result is considered clinically significant, the investigator will

provide details about the action taken with respect to the test drug and about the patient's outcome.

Duration of adverse event collection: The collection and recording of AEs in the database will begin on the first day of initiation of the study drug and will continue for 30 days after the 3 month landmark visit for both arms, after which the events will be captured in the medical record but not abstracted or recorded in the database or toxicity tables.

All Serious Adverse Events will be collected and recorded in the database from the first day of initiation of the study drug and will continue for 30 days after the 3 month landmark visit for both arms. Serious Adverse Event recording for only that are definitely, probably or possibly related to the study drug will continue as long as the subject remains on study. AEs will be followed until satisfactory resolution as long as the subject remains on study.

8.4 Serious Adverse Events Characterization

Serious adverse events will be attributed as definitely (clearly related to the research), probably (likely related to the research), possibly (may be related to the research), unlikely (doubtfully related to the research) and unrelated (clear not related to the research).

TRSAEs are those attributed as definitely or probably. As detailed in section 7.6 stopping rules, TRSAE that will be monitored and considered for early stopping the study according to statistically determined criteria include Death and any grade IV toxicity considered to be probably or definitely related to study medication.

Hospitalizations for administrative issues (e.g., to receive a transfusion after hours) or movement to the ICU for routine monitoring per administrative requirements or nosocomial isolation will not be considered as an SAE.

8.5 NIH Intramural IRB and NHLBI Clinical Director (CD) Reporting

Expedited Reporting

Events requiring expedited reporting will be submitted to the IRB per HRPP Policy 801 "Reporting Research Events".

Reports to the IRB at the time of Continuing Review (CR):

The PI or designee will refer to HRPP Policy 801 "Reporting Research Events" to determine IRB reporting requirements.

Reports to the CD:

The PI or designee will refer to NHLBI DIR guidelines to determine CD reporting requirements and timelines.

8.6 Reporting of Pregnancy

Subjects who become pregnant during the study should discontinue the study drug (Sirolimus) immediately. The investigator, or his/her designee, will collect pregnancy information on any subject who becomes pregnant while participating in this study. The investigator, or his/her designee, will submit pregnancy information to the NHLBI Clinical Director within two weeks of learning of a subject's pregnancy. While pregnancy itself is not considered to be an AE or SAE, any pregnancy complication or elective termination of a pregnancy for medical reasons will be recorded and reported the IRB as an AE or SAE. A spontaneous abortion is always considered to be an SAE and will be reported. Furthermore, any SAE occurring as a result of a post-study pregnancy and is considered reasonably related to the investigational product by the investigator, will be reported. While the investigator is not obligated to actively seek this information in former study participants, he/she may learn of an SAE through spontaneous

reporting.

8.7 Sample Collection, Storage, Biospecimen and Data Management Plan

Biospecimen Management: Specimens and their derivatives (e.g., genomic material, cell lines) will be coded and stored in conformity with DIR Policy (e.g., BSI). Coded biospecimens may be sent to collaborators outside of the NIH with IRB approval in accordance with applicable NIH and DIR Policy for sharing research resources, including an executed material transfer agreement. Biospecimens with subject personal identifiers may be sent to associate investigators and collaborators outside of the NIH only after approvals of all applicable IRBs, an executed reliance agreement with NIH's IRB, or an extension of the NIH's FWA through an Individual Investigator Agreement.

Data Management: The principal investigator, associate investigators, research nurses and/or a data manager will assist with the data management efforts. Data will be abstracted from Clinical Center progress notes as well as intake forms and the case report forms. Laboratory data from NIH will be reviewed using CRIS. The principal investigator, associate investigators/research nurses and/or a contracted data manager will assist with the data management efforts to ensure that data is verifiable and evaluable. Data will be abstracted from Clinical Center progress notes as well as from progress notes forwarded from the subjects' home physician.

The PI will be responsible for overseeing entry of data into an in-house password protected electronic system and ensuring data accuracy, consistency and timeliness. Laboratory values from referring home physicians will be entered into the system. The principal investigator, associate investigators/research nurses and/or a contracted data manager will assist with the data management efforts to ensure that data is verifiable and evaluable.

Research data will be prospectively collected by authorized Investigator personnel and entered into an NHLBI approved Database. The database is a web-based, 21 CFR 11 compliant database which will consist of the study specific set of electronic CRFs (e-CRFs) used for capturing, managing and reporting clinical research data.

The database will maintain complete data records on each research subject including:

- White blood cell count
- Absolute lymphocyte count (percentage if absolute not available)
- Absolute neutrophil count (percentage if absolute not available)
- Absolute reticulocyte count, if available (percentage if absolute not available, then also need the red blood cell count)
- Hemoglobin
- Platelets

Subjective and objective patient experiences during the duration of the study will be documented in the patient medical record notes. These protocol notes will serve as the primary source material from which data will be collected in the Database. Any pertinent supplementary information obtained from outside laboratories, outside hospitals, radiology reports, laboratory reports, or other patient records will be used as additional source for data collection.

We will maintain the confidentiality of identifiable private information collected in this Clinical Trial and protect the privacy of the individual human subjects. Primary data containing individually identifiable information obtained during the conduct of the protocol will be kept in secure network drives or in approved alternative sites that comply with NIH information security standards. Neither individual personal identifiers nor the key linking coded data to individuals will be released without prior IRB approval and an executed CDA or MTA. Identifiable data will

not be sent outside NIH without prior IRB approval or appropriate conditions for disclosure outlined in the executed CDA or MTA.

Storage: All samples will be stored in the laboratory of Dr. Young. Collected samples will be de-identified prior to storage in the laboratory of the principal investigator following current NHLBI DIR BSI Policy. Efforts to ensure protection of patient information include;

- Each sample is assigned a unique number.
- Vials holding patient samples are labeled with the sequential laboratory accession ID number that does not contain any personal identifier information.
- An electronic database is used to store patient information related to the coded samples
- The laboratory is located in a controlled access building and laboratory doors are kept locked. Visitors to the laboratory are required to be accompanied by laboratory staff at all times.
- Hard copy records or electronic copies of documents containing patient information are kept in the locked laboratory or other controlled access locations.

Tracking: Samples will be ordered and tracked through the CRIS Research Screens. Should a CRIS screen not be available, the NIH form 2803-1 will be completed and will accompany the specimen and be filed in the medical record. Samples will not be sent outside NIH without an executed MTA or CTA. Identifiable samples will not be sent outside NIH without prior IRB approval.

End of study procedures: Data will be stored in locked cabinets and in a password protected database until it is no longer of scientific value. At the completion of the protocol (termination), samples and data will be maintained in a repository for future research.

Loss or destruction of data: Should we become aware that a major breech in our plan to protect patient confidentiality and trial data has occurred, the IRB will be notified.

Publication Policy: Given the research mandate of the NIH, patient data including the results of testing and responses to treatment will be entered into an NIH-authorized and controlled research database. Any future research use will occur only after appropriate human subject protection institutional approval such as prospective NIH IRB review and approval or an exemption from the NIH Office of Human Subjects Research Protections (OHSRP).

Privacy and Confidentiality: All efforts, within reason, will be made to keep subjects' protected health information (PHI) and private identifiable information (PII) private. Using or sharing ("disclosure") such data must follow federal privacy rules. Under certain circumstances, the United States Office of Human Research Protections (OHRP), The US Food and Drug Administration (FDA), and the Institutional Review Board (IRB), will be able to inspect and copy confidential study-related records which identify participants by name. Therefore, absolute confidentiality cannot be guaranteed.

***Data sharing and future use of data*:** Research data may be shared with qualified non-collaborator recipients following publication of the primary research results after removal of PII and IRB approval. Future research use of data not defined in the research protocol may occur only after IRB review and approval or an exemption from the NIH Office of Human Subjects Research Protections (OHSRP). Refusal of a research subject participant to permit future use of data--other than required in the protocol or by the FDA--will be honored. Limitations in data sharing and future use of data due to contractual obligations (e.g., CRADAs) or intellectual property proceedings (such as patent filings) will be honored.

***Future use of biospecimens*:** Following analyses of biospecimens for primary research purposes, remaining samples suitable for future research will be stored in manner that conforms with DIR

policy (such as BSI) or in a publicly accessible research biospecimen repository following IRB or OHSRP approval, as applicable. Biospecimens may be destroyed only when permitted by the clinical director and the IRB.

Any future research use of biospecimens not defined in the protocol in which NHLBI investigators are engaged in research (e.g., they are undertaking research activities and hold the key that identifies research subjects) requires IRB review and approval. Refusal of a research subject participant to allow for future use of identifiable biospecimens--other than required in the protocol or for appropriate regulatory purposes, e.g., by the FDA--will be honored.

Sharing of Biospecimens with Non-NIH Investigators: Coded biospecimens (NHLBI investigators hold the key that identifies research subjects) to be shared outside of NIH for future research use requires IRB review and approval (for research collaborations where results will be returned and re-identified) and an executed Material Transfer Agreement (MTA). Unlinked biospecimens (no key to identify research subjects exists) to be shared outside of NIH for future research use requires an MTA. There are a few types of biospecimens that do not require IRB approval for future research use outside of NIH, such as specimens from deceased individuals (refer to OHSRP SOP5, Appendix 1 for complete list); an executed transfer agreement is required in these special cases.

9.0 Human Subject Protection

9.1 Rationale for Subject Selection

This study will be open to all patients who fit the inclusion criteria and provide informed consent to protocol participation. No subjects will be excluded from participation based on gender, race or ethnicity. Epidemiologic studies suggest that the gender will be approximately evenly split between male and females, and that 90% of the patients will be Caucasian. However previous experience at our institution suggests that distribution will be:

- *By gender*: Approximately 60% males and 40% females;
- *By race/ethnicity*: Approximately 55% White, 15% Black, 6% Asian and 24% Hispanic;
- *By age*: Likely to range between 2 and 82 (median age of 30); roughly 20% of patients will be under the age of 18.

Justification for inclusion of cognitively impaired subjects: This research provides the prospect of direct benefit; therefore, inclusion is justified. The benefits to the participants could be improvement of cytopenias resulting in improved quality of life and also decreased morbidity and mortality from transfusion-associated viral agents, iron overload, and/or a susceptibility to infections. Potentially, treatment with other more toxic therapies could also be avoided or postponed. Not allowing participants who cannot provide consent would deny them the potential benefits this protocol offers for their AA. There are no plans to include institutionalized participants.

Recruitment efforts:

The recruitment strategies to be used for this study may include:

- Clinicaltrials.gov website,
- Clinical Center Research Studies (“Search the Studies”) website,
- The Aplastic Anemia Foundation website,
- National Heart, Lung and Blood Institute (NHLBI) patient recruitment website,
- Social media postings such as Facebook and Clinical Center Twitter,
- Use of Clinical Center Office of Patient Recruitment Services (OPR) including OPR Listservs *(Email list of those interested in receiving study recruitment updates.) (Links to specific study page on clinicaltrials.gov)*. Includes the OPR Protocols and OPR Healthy Volunteers listservs and 3 NIH listservs (NIH Post back, NIH Clinical Fellows & NIH

Study Volunteers),
- ResearchMatch,
- NIH Newsletters.

If recruitment goals are not met, additional recruitment strategies will be developed by the Clinical Center Office of Patient Recruitment. Hematologists and Oncologists throughout the country will be informed about the protocol by letter. Because many aplastic anemia patients may respond to initial immunosuppressive treatment with a response that is sufficient to prevent serious infections, but have persistent thrombocytopenia, we will also be able to rapidly recruit study patients who have completed other trials for aplastic anemia therapy within the Branch.

Reimbursement for protocol participation, travel, food, and lodging will be consistent with NIH guidelines. In determining reimbursement, the following factors are considered applicable to this protocol: the patients are diagnosed with a rare disease; the patient population is sick; the protocol offers the potential for direct benefit; the protocol regimen is demanding; and in order to complete accrual in a reasonable timeframe a geographically dispersed participant population is required.

Payment for participation: $0. The study participants will not be reimbursed for their time and inconvenience.

Competition between Branch Protocols: There are no competing Branch protocols for this patient population.

9.2 Risks in Relation to Benefit

For Adult Subjects

The research has a prospect of direct benefit to individual subjects, and is likely to yield generalizable knowledge about the subject's disorder or condition. The benefits to the participants could be improvement of cytopenias resulting in improved quality of life and also decreased morbidity and mortality from transfusion-associated viral agents, iron overload, and/or a susceptibility to infections. Potentially, treatment with other more toxic therapies could also be avoided or postponed.

For Pediatric Subjects

Pediatric subjects are included because:

(a) the risk is justified by the anticipated benefit to the subjects: We are offering pediatric subjects at risk of relapse of a lethal hematological disease a potential disease-modifying therapy.
(b) the relation of the anticipated benefit to the risk is at least as favorable to the subjects as that presented by available alternative approaches. Potentially, treatment with other more toxic therapies could also be avoided or postponed.

For Cognitive Impaired subjects

This research provides the prospect of direct benefit; therefore, inclusion is justified. The benefits to the participants could be improvement of cytopenias resulting in improved quality of life and also decreased morbidity and mortality from transfusion-associated viral agents, iron overload, and/or a susceptibility to infections. Potentially, treatment with other more toxic therapies could also be avoided or postponed.

9.3 Participation of Very Young Children

In principle, age is not a consideration. But in practice, we are limiting the protocol to subjects who are age 2 years and older because our clinic does not have the expertise to care for infants. In

addition, per Clinical Center guidelines, we are limiting participation to children who weigh >12 kg.

9.4 Exclusion of Pregnant Women and Nursing Mothers

Adverse effects have been observed in animal reproduction studies with sirolimus. Therefore, women of childbearing potential must agree to use adequate contraception (hormonal or barrier method of birth control; abstinence) prior to and for 12 weeks following treatment with sirolimus. If a female patient becomes pregnant or suspects she is pregnant while on study, her treating physician should be immediately informed.

10.0 Informed Consent

Informed consent will be conducted following OHSRP Policy 301- Informed Consent.

An IRB-approved consent form will be provided to the participant electronically or by hard copy for review prior to consenting. The initial consent process as well as re-consent, when required, may take place in person or remotely (e.g., via telephone or other NIH approved platforms). The investigational nature and objectives of this trial, the procedures, and their attendant risks and discomforts and potential benefits will be carefully explained to the participant in a private setting. The participant will be given as much time as they need to review the document and to consult with their family, friends, and personal health care providers. In addition, a study team member will be available to answer any questions.

A signed and dated informed consent document will be obtained by any investigator authorized to consent (See Key Study Personnel Page) prior to entry onto the study. Consent may be obtained with required signatures on the hard copy of the consent or on the electronic document.

When a document that is in electronic format is used for obtaining consent, this study may use the iMed platform which is 21 CFR, Part 11 compliant, to obtain the required signatures. During the consent process, participants and investigators may view the same approved consent document simultaneously when participant is being consented in person at the Clinical Center or both may view individual copies of the approved consent document on screens in their respective locations remotely. Signatures may be obtained either by both directly signing on the device that the consenting investigator is using (when in person) or through iMed Mobile Signature Capture (remotely) which allows texting or emailing a link to the participant. That link allows the participant to review the consent, then proceed to sign on the device they are using.

Whether hard copy or electronic, both the investigator and the participant will sign the document with a hand signature using a pen (if using hard copy), finger, stylus, or mouse (if electronic).

When done remotely, if the participant prefers to sign a hard copy, they may be instructed to sign and date the consent document during the discussion and mail, secure email or fax the signed document to the consenting investigator.

Whether in person or remotely, the privacy of the participant will be maintained.

Finally, the fully signed informed consent document will be stored in the electronic medical record, and the participant will receive a copy of the signed informed consent document.

If the patient is a minor, the parent who signs the consent for the minor must be a legally recognized parent or guardian. Where deemed appropriate by the clinician, and the child's parent or guardian, the child will also be included in all discussions about the trial and a minor's assent will be obtained. The parent or guardian will sign on the designated line on the informed consent

attesting to the fact that the child had given assent. In cases where parents share joint legal custody for medical decisions for a child (e.g., by custody agreement or court order), both parents must give their permission regardless of the level of risk of the research. Exceptions may be made if one parent has since died, become incompetent, or is not reasonably available (e.g., incarcerated).

In addition, if any of the parents/legal guardian of a potential minor participant (including parents who share custody of the minor) are unable to be physically present at the NIH at the time of consent to this protocol, the telephone/telehealth consent process will be followed.

When a pediatric subject reaches age 18, continued participation will require re-consenting of the now adult with the standard protocol consent document to ensure legally effective informed consent has been obtained. Should sample or data analysis continue following completion of active participation and the subject has reached 18 years of age, we will attempt to contact the subject using the last known contact information to obtain consent for continued use of data or samples collected during their prior visit. Given the length of time that may have transpired for some of the subjects since their last visit for this study, we request waiver of informed consent for those individuals who after good faith efforts to contact them, we are unable to contact.

Requirements for Waiver of Consent consistent with 45 CFR 46.116 (d), each of which must be addressed in relation to the protocol:

(1) The research involves no more than minimal risk to the subjects;
   a) Analysis of samples and data from this study involves no additional risks to subjects.
(2) The waiver or alteration will not adversely affect the rights and welfare of the subjects;
   a) Samples and data will be kept in secure locations in the laboratory of Dr. Young. Retention of samples or data does not affect the welfare of subjects.
(3) The research could not practicably be carried out without the waiver or alteration; and
   a) Considering the length of time between a minor's enrollment and their age of majority, it is possible that more than a few subjects may be lost to follow up. A significant reduction in the number of samples analyzed could impact the quality of the research.
(4) Whenever appropriate, the subjects will be provided with additional pertinent information after participation.
   a) We only plan to request a waiver of reconsent for those subjects who have been lost to follow-up.

Informed Consent for adult research participants unable to provide consent:
If there is an unexpected enrollment of a research participant unable to provide informed consent, we will follow the procedures for obtaining informed consent from a legally authorized representative (LAR) (Risk Level B) per NIH HRPP Policy 403.

Consent and Assent:
Procedures to determine capacity: If documentation of decision making capacity is not present in the medical record or the investigator questions the decision-making capacity of the individual, then the Ability to Consent Assessment Team (ACAT) (301-496-9675 or 301-496-2429) will be contacted to make the determination.

Procedures to obtain assent and documentation of assent or dissent: The informed consent discussion will include the individual unable to provide informed consent along with LAR. The individual unable to provided informed consent will be asked if they agree to participate in the research and this will be documented in the medical record.

11.0 Risks and Discomforts

11.1 Related to Sirolimus

Sirolimus induces immune suppression, which is associated with opportunistic infection and an increased rate of malignancy, particularly skin cancer. Some individuals develop hypersensitivity to sirolimus. May cause an increase in serum cholesterol and triglycerides, which may be associated with pancreatitis. Impaired renal function may ensue from long-term administration.

The anticipated toxicities of sirolimus in this trial are those related to its immunosuppressive properties, such as an increased likelihood of infection, and mucosal (including mouth, gastric, small bowel, or large bowel) ulcers, which may bleed. Other possible toxicities are listed here and include those reported with $\geq$ 3% and <20% incidence in patients in any Sirolimus treatment group in the two controlled clinical trials for the prevention of acute organ graft rejection:

Body as a Whole: abdomen enlarged, abscess, ascites, cellulitis, chills, face edema, flu syndrome, generalized edema, hernia, Herpes zoster infection, lymphocele, malaise, pelvic pain, peritonitis, sepsis;

Cardiovascular System: atrial fibrillation, congestive heart failure, hemorrhage, hypervolemia, hypotension, palpitation, peripheral vascular disorder, postural hypotension, syncope, tachycardia, thrombophlebitis, thrombosis, vasodilatation;

Digestive System: anorexia, dysphagia, eructation, esophagitis, flatulence, gastritis, gastroenteritis, gingivitis, gum hyperplasia, ileus, liver function tests abnormal, mouth ulceration, oral moniliasis, stomatitis;

Endocrine System: Cushing's syndrome, diabetes mellitus, glycosuria, hypercholesterolemia, hyperlipidemia;

Hematologic and Lymphatic System: ecchymosis, leukocytosis, lymphadenopathy, polycythemia, thrombotic thrombocytopenic purpura / hemolytic-uremic syndrome, leukopenia;

Metabolic and Nutritional: acidosis, alkaline phosphatase increased, BUN increased, creatine phosphokinase increased, dehydration, healing abnormal, hypercalcemia, hyperglycemia, hyperphosphatemia, hypocalcemia, hypoglycemia, hypomagnesemia, hyponatremia, lactic dehydrogenase increased, SGOT increased, SGPT increased, weight loss;

Musculoskeletal System: arthrosis, bone necrosis, leg cramps, myalgia, osteoporosis, tetany:

Nervous System: anxiety, confusion, depression, dizziness, emotional lability, hypertonia, hypesthesia, hypotonia, insomnia, neuropathy, paresthesia, somnolence;

Respiratory System: dyspnea, changes in PFTs, asthma, atelectasis, bronchitis, cough increased, epistaxis, hypoxia, lung edema, pleural effusion, pneumonia, rhinitis, sinusitis, diffuse alveolar hemorrhage;

Skin and Appendages: fungal dermatitis, hirsutism, pruritus, skin hypertrophy, skin ulcer, sweating;

Special Senses: abnormal vision, cataract, conjunctivitis, deafness, ear pain, otitis media, tinnitus;

Urogenital System: albuminuria, bladder pain, dysuria, hematuria, hydronephrosis, impotence,

kidney pain, kidney tubular necrosis, nocturia, oliguria, pyelonephritis, pyuria, scrotal edema, testis disorder, toxic nephropathy, urinary frequency, urinary incontinence, urinary retention.

Increased Serum Cholesterol and Triglycerides: The use of sirolimus in renal transplant patients was associated with increased serum cholesterol and triglycerides that may require treatment. Patients in the sirolimus arm will receive treatment for new-onset hypercholesterolemia and hypertriglyceridemia with lipid-lowering agents if clinically indicated under the discretion of the primary investigator.

Skin cancer: Patients on immunosuppressive therapy are at increased risk for skin cancer. Patients in the sirolimus arm will be advised about limiting exposure to sunlight and UV light be wearing protective clothing and using a sunscreen with a high protection factor.

Less frequently occurring adverse events included: mycobacterial infections, Epstein-Barr virus infections, BK virus-associated nephropathy, skin cancer, lymphoma, pericardial effusion, posterior reversible encephalopathy syndrome (PRES), and pancreatitis.

11.2 Related to bone marrow aspirate and biopsy

No major risks are involved with bone marrow aspirate and biopsy. However, a small risk of infections, pain, bleeding, and hematoma formation at the site of the aspiration exists with the procedure.

11.3 Related to blood draws

No major risks are involved with blood draws. Minor complications including bleeding, pain, and hematoma formation at the site of blood draws, vasovagal reactions or infections may rarely occur.

12.0 Pharmaceuticals

12.1 Sirolimus (Rapamune)

12.1.1 Supply

For patient administration, oral tablets and liquid formulation will be purchased by the NIH clinical Center pharmacy department from commercial sources.

12.1.2 Storage and Stability

Sirolimus is available for administration as an oral solution containing 1 mg/mL sirolimus. Sirolimus is also available as a white, triangular-shaped tablet containing 1-mg sirolimus, and as a yellow to beige triangular-shaped tablet containing 2-mg sirolimus.

Rapamune® Tablets should be stored at 20° to 25°C (USP Controlled Room Temperature) (68° to 77°F). Use cartons to protect blister cards and strips from light. Dispense in a tight, light-resistant container as defined in the USP.

Rapamune® Oral Solution bottles should be stored protected from light and refrigerated at 2°C to 8°C (36°F to 46°F). Once the bottle is opened, the contents should be used within one month. If necessary, the patient may store the bottles at room temperatures up to 25°C (77°F) for a short period of time (e.g., not more than 15 days for the bottles). An amber syringe and cap are provided for dosing and the product may be kept in the syringe for a maximum of 24 hours at room temperatures up to 25°C (77°F) or refrigerated at 2°C to 8°C (36°F to 46°F). The syringe should be discarded after one use. After dilution, the preparation should be used immediately. Rapamune Oral Solution provided in bottles may develop a slight haze when refrigerated. If such a haze occurs allow the product to stand at room temperature and shake gently until the haze disappears. The presence of this haze does not affect the quality of the product.

12.1.3 Administration

Oral tablets should be administered approximately four hours after cyclosporine administration. Because food, in particular fatty foods, can decrease the absorption of sirolimus, the tablets or oral solution should be administered between meals (lunch and dinner).

Oral Solution

The amber oral dose syringe should be used to withdraw the prescribed amount of Rapamune® Oral Solution from the bottle. Empty the correct amount of Rapamune from the syringe into only a glass or plastic container holding at least two (2) ounces (1/4 cup, 60 mL) of water or orange juice. No other liquids, including grapefruit juice, should be used for dilution. Stir vigorously and drink at once. Refill the container with an additional volume (minimum of four [4] ounces [1/2 cup, 120 mL]) of water or orange juice, stir vigorously, and drink at once.

12.1.4 Regulatory Status:

Off Label Usage

Sirolimus will be used in this study beyond what is indicated in the package insert. Sirolimus is approved by the FDA as an immunosuppressive agent for the prophylaxis of organ rejection in patients aged ≥ 13 years receiving renal transplants, in renal patients at high immunologic risk, and in lymphangioleiomyomatosis. The use of this drug meets the requirements for an exemption from the IND regulations, 21 CFR 312, specifically:

1. The investigational drug is lawfully marketed in the United States
2. The investigation is not intended to be reported to the FDA as a well-controlled study in support of a new indication for use of the drug product
3. The investigation is not intended to support a significant change in advertising to an existing lawfully marketed prescription drug product
4. The investigation does not involve a route of administration or dosage level or use in a patient population or other factor that significantly increases the risks (or decreases the acceptability of the risks) associated with the use of the drug product.
5. The investigation will be conducted in compliance with the requirements for institutional review set forth in FDA regulations 21 CFR 56, and requirements for informed consent as set forth in FDA regulations 21 CFR 50
6. The investigation will be conducted in compliance with FDA regulations 21 CFR 312.7: Promotion and charging for investigational drugs.

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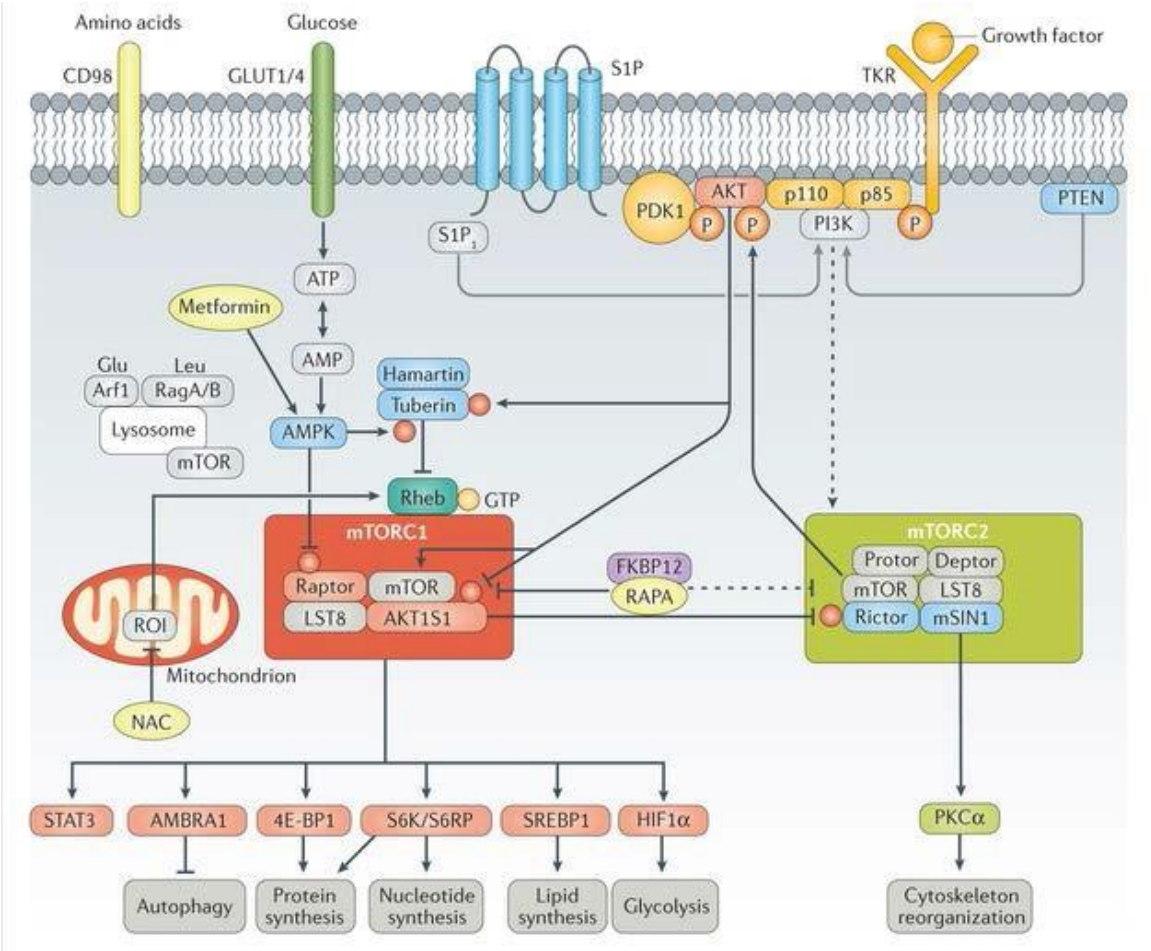
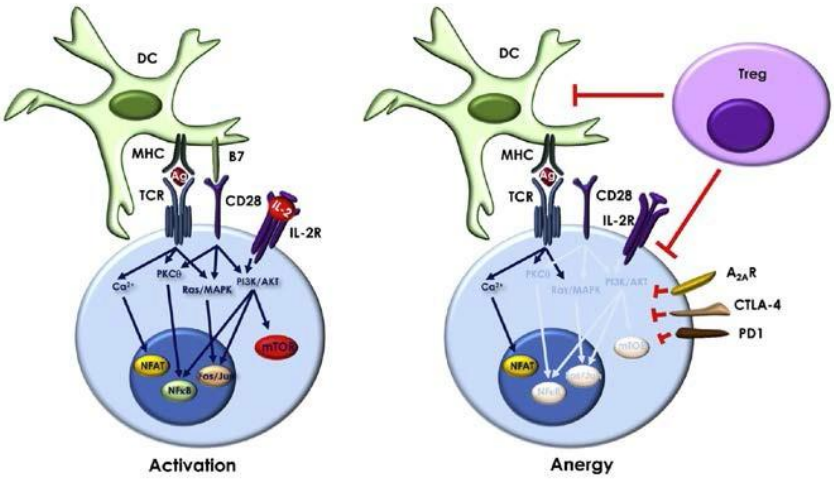
14.0 APPENDIX A: Supplemental Figures





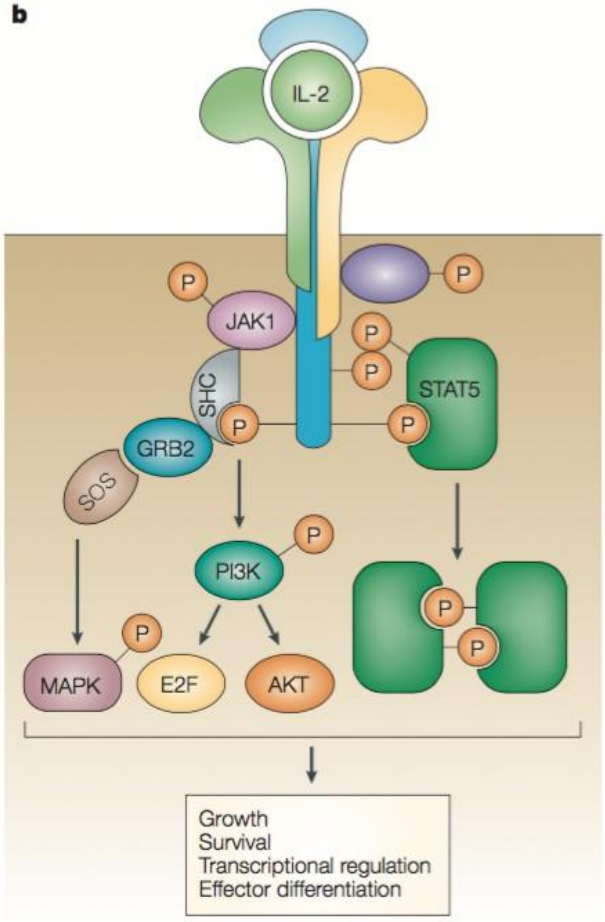


Figure 1 | **IL-2 receptor signalling. a** | The high-affinity interleukin-2 receptor (IL-2R) consists of three subunits: the α-chain of the IL-2R (IL-2Rα; also known as CD25), the β-chain of the IL-2R (IL-2Rβ; also known as CD122) and the common cytokine-receptor γ-chain (γc; also known as CD132). It is thought that in the absence of IL-2, these subunits are not pre-assembled on the surface of an IL-2R-expressing T cell. **b** | The binding of IL-2 to IL-2Rα drives the association of this subunit with IL-2Rβ and the γc to form a stable heterotrimer, which then leads to the initiation of signal transduction. Janus activated kinase 3 (JAK3) molecules that are associated with the γc, and JAK1 molecules that are associated with IL-2Rβ, phosphorylate key tyrosine residues in the cytoplasmic tail of IL-2Rβ, the γc and the JAK molecules themselves. This amplifies the association of these tyrosine kinases and induces the association of the adaptor SHC (SRC-homology-2-domain-containing transforming protein C), and either signal transducer and activator of transcription 5 (STAT5) or STAT3, with the cytoplasmic tail of IL-2Rβ. The complexed SHC allows activation of the mitogen-activated protein kinase (MAPK)- and phosphatidylinositol 3-kinase (PI3K)–AKT-signalling pathways. Tyrosine residues in STAT5 are also phosphorylated, leading to the dimerization of STAT5 and its translocation to the nucleus where it regulates STAT5-responsive genes. GRB2, growth-factor-receptor-bound protein 2; SOS, son of sevenless homologue.

17.0 APPENDIX B: Schedule of Events

Activity	Screening	Baseline	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	Month 3 (12 Weeks)	Year 1	Year 2	Year 3	Year 4	Year 5	Q 12 weeks (Yr. 1 to 5)	At time of Relapse
Informed consent		X																			
Medical history	X	X												X	X	X	X	X	X		
Physical exam	X	X												X	X	X	X	X	X		
Vital signs	X	X												X	X	X	X	X	X		
Concomitant med assessment#	X	X												X	X	X	X	X	X		
CBC with differential***	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
Reticulocyte count	X	X	X*	X*	X*	X*	X*	X*	X*	X*	X*	X*	X*	X							
Direct antiglobulin test	X[$]	X																			
Human Chorionic Gonadotropin/ Preg. test urine	X	X												X							
Acute care, mineral and hepatic panel	X	X				X				X				X	X	X	X	X	X		
Iron and transferrin		X																			
Ferritin		X																			
PNH Flow Analysis, WBC		X												X**	X	X	X	X	X		
PNH Flow Analysis, RBC		X												X**	X	X	X	X	X		
Lymphocyte phenotyping TBNK		X												X							
Fasting lipid panel		X				X				X				X	X						
Fasting glucose		X												X[$]	X[$]						
Hgb A1C		X												X[$]							
Protein creatinine Ratio, urine		X												X[$]	X[$]						

Activity	Screening	Baseline	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	Month 3 (12 Weeks)	Year 1	Year 2	Year 3	Year 4	Year 5	Q 12 weeks (Yr. 1 to 5)	At time of Relapse
Albumin/Creatinine Ratio, urine		X												X[$]	X[$]						
Protein, total, Other - urine		X												X[$]	X[$]						
Creatinine, Random Urine		X												X[$]	X[$]						
Albumin, urine		X												X[$]	X[$]						
Anti CMV IgG, IgM antibody		X																			
Anti-HAV Antibody, total		X																			
HBs Ag, screening		X																			
Anti HBs antibody		X																			
Anti HCV antibody		X																			
Anti HSV Type 1/2 antibody serum		X																			
Anti EBV antibody Panel		X																			
Anti-HIV-1/2 Antibody		X																			
CMV/EBV PCR; Quantitative, blood		X																			
Sirolimus level (SRL arm only)[β]			X	X	X	X		X		X		X		X							
Research blood		X												X**	X	X	X	X	X		X
Research bone marrow aspirate		X												X		X	X	X	X		
Bone marrow aspiration and core biopsy		X														X[$]	X[$]	X[$]	X[$]		
Cytogenetics on BM		X														X[$]	X[$]	X[$]	X[$]		

* Reticulocyte count is optional at these time points
[$] Performed at PI's Discretion
[#] Recorded in the source documents but not entered into the database
** These tests are performed at the NIH and are optional.

*** CBC with differential and reticulocyte count (if available) at least every 2 weeks (+/- 4 days) for 3 months (12 weeks)"
Note: Landmark 12 months (+/- 30 days), 36 months (+/- 90 days), 48 months (+/- 90 days), and 60 months (+/-90 days) may be conducted at the local physician's office along with a telehealth visit with a provider of the research team (PI/AI).
[β] Post loading sirolimus dose may be collected