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Long-term follow up of IPEX syndrome patients after different therapeutic strategies:
an international multicenter retrospective study

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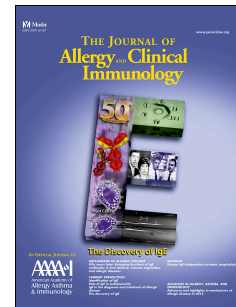
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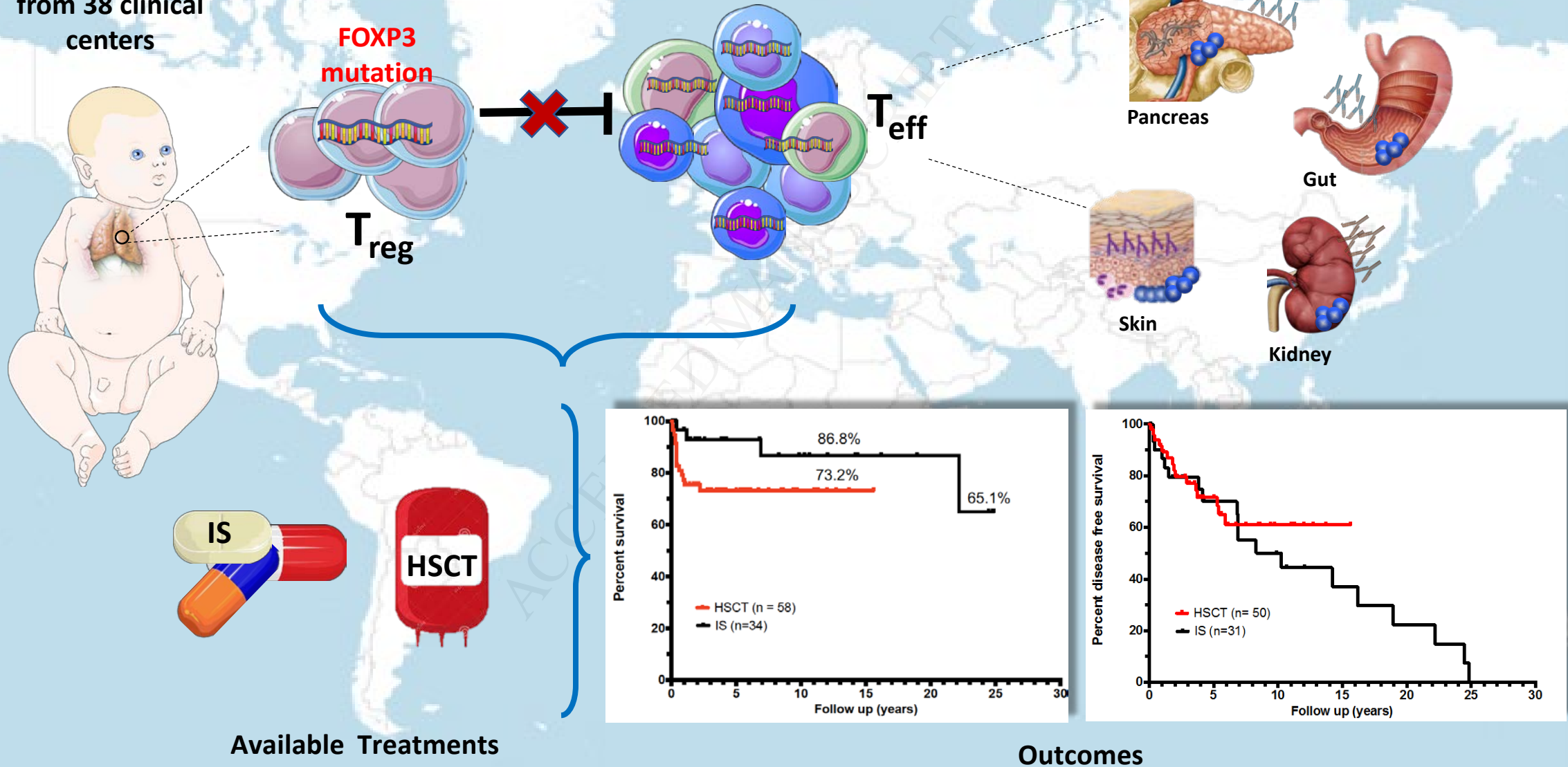
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96 IPEX patients
from 38 clinical
centers



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an international multicenter retrospective study**

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Key words: IPEX, Primary Immune Deficiency, FOXP3, Treg cells, hematopoietic stem cell transplantation, immunosuppression, rapamycin, enteropathy, neonatal diabetes, genetic autoimmunity.

Abbreviations:

Alm, Alemtuzumab
 ATG, anti-thymocyte globulin
 CSA, cyclosporine
 DFS, Disease Free Survival
 FK506, tacrolimus
 FKH, forkhead domain
 Full, full conditioning regimen
 GvHD, Graft-versus-Host Disease
 HAA, anti-harmonin auto-antibodies
 HSC, hematopoietic stem cells
 HSCT, hematopoietic stem cell transplantation
 IPEX, Immunodysregulation Polyendocrinopathy Enteropathy X-linked
 IS, immunosuppression
 Minimal Intensity Conditioning (MIC
 MMF, mycophenolate mofetil
 MTX, methotrexate
 OI, organ involvement
 OS, Overall Survival
 PB, peripheral blood
 PHA, phytohemagglutinine
 RIC, Reduced Intensity Conditioning
 RIT, reduced intensity transplant
 T1D, Type 1 Diabetes
 TNC, total nucleated cells
 Treg cells, regulatory T cells
 TSDR, Treg cell-specific demethylated region
 VAA, anti-villin auto-antibodies

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ABSTRACT

Background. Immunedysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome is a monogenic autoimmune disease caused by *FOXP3* mutations. Because it is a rare disease, the natural history and response to treatments, including allogeneic hematopoietic stem cell transplantation (HSCT) and immunosuppression (IS), have not been thoroughly examined.

Objective. To evaluate disease onset, progression and long-term outcome of the two main treatments in long-term IPEX survivors.

Methods. Clinical histories of 96 patients with a genetically proven IPEX syndrome were collected from 38 institutions worldwide and retrospectively analyzed. To investigate possible factors suitable to predict the outcome, an organ involvement (OI) scoring system was developed.

Results. We confirm neonatal onset with enteropathy, type 1 diabetes (T1D), and eczema. In addition, we found less common manifestations in delayed onset patients or during disease evolution. There is no correlation between the site of mutation and the disease course or outcome, and the same genotype can present with variable phenotypes. HSCT patients (n=58) had a median follow-up of 2.7 years (range: 1 week - 15 years). Patients receiving chronic IS (n=34) had a median follow-up of 4 years (range: 2 months - 25 years). The overall survival (OS) after HSCT was 73.2% (95% confidence interval [CI], 59.4 to 83.0) and after IS was 65.1% (95 % CI, 62.8 to 95.8). The pre-treatment OI score was the only significant predictor of OS after transplant (p=0.035) but not under IS.

Conclusions. Patients receiving chronic IS were hampered by disease recurrence or complications, impacting long-term disease-free survival. When performed in patients with a low OI score, HSCT resulted in disease resolution with better quality of life, independent of age, donor source or conditioning regimen.

Capsule summary. This international retrospective multicenter study of patients with long-term IPEX syndrome (n=96) provides data on onset, disease progression, and outcomes after different treatments to inform future therapeutic choices.

Key messages

- Pre-treatment organ impairment score in IPEX best predicts overall survival after HSCT.
- HSCT and IS recipients experience similar overall survival, but those receiving HSCT demonstrate higher rates of disease-free survival.

INTRODUCTION

The Immune dysregulation-polyendocrinopathy-enteropathy-X-linked (IPEX) syndrome is a primary immunodeficiency caused by hemizygous mutations in the gene *FOXP3*, which encodes an essential transcription factor required for maintenance of immunologic tolerance by thymus-derived regulatory T (Treg) cells. Since its first clinical description in 1982¹ and its genetic characterization in 2001^{2,3}, IPEX syndrome has gathered the attention of scientists and physicians as the prototype of a monogenic autoimmune disease and immune deficiency affecting the immune regulatory compartment^{4,5}. Significant advances have been made in elucidating the complex disease pathogenesis⁶⁻⁹. The typical manifestations of severe enteropathy, type 1 diabetes (T1D) and eczema have been extensively reported, but IPEX still poses a significant therapeutic challenge. Single reports of young adults affected by “atypical” or “late onset” IPEX forms have suggested heterogeneity in both the clinical presentation and their response to therapy¹⁰⁻¹². Immunosuppressive therapy, the first line treatment for IPEX patients, has changed considerably in recent years with the introduction of new drugs with an immune suppressive and modulatory action. Nevertheless, the use and efficacy of these therapies are largely undocumented in IPEX patients. Currently, the only potentially curative therapy for IPEX syndrome is allogeneic hematopoietic stem cell transplantation (HSCT) with the longest published follow-up of 8 years^{13,14}. Both HLA-identical and matched-unrelated HSCT can be successful. However, while there is a general understanding to treat early, a comprehensive study comparing different transplant protocols and long-term outcomes is not available. In addition, a significant number of patients cannot undergo HSCT, due to limited donor availability and high risk/benefit ratio. Finally, older patients with a mild disease phenotype, in whom the clinical manifestations are often not severe enough to justify HSCT, raise concerns regarding the appropriate treatment¹⁵.

This international multicenter retrospective analysis of IPEX patients aims to provide a comprehensive view of the disease, its evolution and outcomes of different therapeutic strategies. We aim to unveil limitations of case reports or small case series by providing a more comprehensive analysis of a large patients’ cohort. Ultimately, our work aims to improve the diagnosis and the long-term treatment for IPEX syndrome, with the goal of achieving definitive cure, which could apply to other immunodeficiencies with autoimmunity.

METHODS

Patients' cohort and definitions

A retrospective multicenter study was performed, in which data were collected from 38 institutions worldwide. All patients included were diagnosed with IPEX syndrome based on the presence of a *FOXP3* mutation (detected but not specified for 5 patients). Data were collected through a detailed survey. Each center notified to their institutional review board (IRB) and, if required, obtained the approval for sharing the data included in the present study. Data were transferred in a completely de-identified form.

Clinical manifestations were defined as "autoimmune" based on exclusion of other causes, the presence of specific autoantibodies or presence of other pathological findings suggestive of autoimmune etiology, or *ex juvantibus* (positive response to immunosuppressive therapy). Based on the number of organs or systems impaired by the autoimmune damage or by secondary complications, before undergoing immunosuppression (IS) or HSCT, we established an organ involvement (OI) scoring system ranging between 0 and 5; one point was assigned for the presence of each of the following: intractable diarrhea, malnutrition, liver dysfunction, respiratory impairment, kidney dysfunction. The selection of these parameters was dictated by their relevance in affecting disease morbidity and survival. The presence of clinical manifestations was assessed by the caring physicians, according to standard of care definitions.

The effect of IS was considered "beneficial" when the physician observed a decrease (partial benefit) or complete disappearance (benefit) of signs and symptoms of the disease.

Conditioning regimens were defined as fully myeloablative (full) or as reduced intensity transplant (RIT). Full conditioning regimens included busulfan plus cyclophosphamide, busulfan of > 14 mg/kg or cumulative AUC 80-90 mg*h/L (when available) plus fludarabine, or treosulfan. RIT encompassed both Reduced Intensity Conditioning (RIC, e.g. fludarabine plus nonmyeloablative doses of busulfan, treosulfan or melphalan) and Minimal Intensity Conditioning (MIC, e.g. fludarabine plus low dose radiation or cyclophosphamide)¹⁶.

Neutrophil engraftment was defined as the first of 3 consecutive days with neutrophils >1000 per mm³ unsupported; platelet engraftment was defined as the first of 3 days with platelets > 50.000 per mm³ unsupported. A "boost" was an additional infusion of hematopoietic stem cells (HSC) without conditioning, while "second or third" transplants were additional infusions of HSC from a different donor with conditioning. T-cell reconstitution (analyzed at 3, 6, 12 months and at the last available follow-up) was defined as >1000 CD3+ T cells per mm³, >500 CD4+ T cells per mm³ and presence of proliferative response to mitogens (considered as positive when within the normal range of the laboratory). B-cell reconstitution was defined as independence from immunoglobulin replacement therapy. Full donor chimerism was defined as >95% donor cells in peripheral blood (evaluated at 3,

6, 12 months and last follow up). Primary graft failure was defined as absence of donor cells in peripheral blood post-transplant. Secondary graft failure was defined as reduction of neutrophil counts, occurring after engraftment, and absence of donor cells (below 5%), despite normal blood counts. The diagnosis and grading of acute or chronic graft-versus-host disease (GvHD) were based on previously published criteria and scoring system¹⁷.

Evaluation of quality of life was reported by the caring physicians without a standardized scoring system (yes/no questions).

Statistical Analysis

Demographic, disease-related or transplant-related characteristics were reported as frequencies for categorical variables, and median and range for quantitative variables. Associations between variables and disease outcome or type of treatment were compared with the use of the chi-square test (categorical variables) or unpaired t-test (continuous variables).

Probabilities of survival after treatment and disease-free survival (DFS) were computed with the use of the Kaplan-Meier estimator and compared with the Log-rank test (Mantel-Cox). We then used a multivariable analysis to examine risk factors for transplantation outcomes and adjust for confounding factors using the Cox (proportional hazard) regression models with Breslow estimator; a p-value of 0.05 or less was considered statistically significant. The variables considered in the Cox regression to model transplantation outcome were age at transplant, pre-HSCT OI score, donor type, degree of HLA-matching, intensity of conditioning regimen, graft source, and number of CD34 cells infused. For a small cohort of patients transplanted at <1 year, the variables used in the Cox regression were age at HSCT, score, and weight. We also examined DFS with a Cox regression model, where death is treated as a censoring event, using as predictor variables the score pre-HSCT, graft failure, and conditioning.

The patients whose mutations were known (n=91) were grouped in four categories depending on type of mutation: missense, splice site, frameshift or in-frame deletions or insertions; and “others” including mutations in the UTR, promoter or start codon. We furthermore explored each mutation effect on protein function through available algorithms such as PolyPhen-2 (providing predictions for amino acid substitutions) and PROVEAN (providing predictions for amino acid substitutions, insertions and deletions), obtaining a numeric score and a qualitative prediction outcome such as “benign” or “neutral”, and “deleterious” or “probably damaging”. We examined associations among the predicted protein scores and each patient score before HSCT or IS and their survival outcome, by evaluating Spearman correlation coefficient and contingency tables.

RESULTS

Initial clinical manifestations, disease course and genetics

Ninety-six patients with *FOXP3* mutations were included in our study. The median age at disease onset was 2 months (ranged from birth to 11.3 years): 41% had onset within the first month of life, 46% between 1 month and 1 year, 10% after 1 year of age, and for 3% of patients the time of onset was unavailable. The median time to diagnosis (elapsed time between onset of symptoms and genetic diagnosis) was 14 months (up to a maximum of 23.9 years) (**Fig 1A**); however, the number of IPEX patients diagnosed per year shows an increased trend (**Fig 1B**).

The most common presentation suggestive of the diagnosis of IPEX syndrome remained the classical triad of enteropathy, T1D and eczema for the majority of the 39/96 patients who had neonatal onset. Gastrointestinal involvement and eczema, but not T1D, were the dominant features of disease presentation after 1 month of age. However, failure to thrive was a hallmark of the disease and it was sometimes the sole initial manifestation after 1 month of age. After 1 year of age the disease could also present with nephropathy or hepatitis, otherwise considered atypical at onset (**Fig 1C, D**).

The symptoms observed over the course of the disease and their prevalence are listed (**Fig 1D**): with the exception of diarrhea, eczema and failure to thrive, nephropathy (autoimmune or secondary to malnutrition and medication) was the most common (33/96), varying from lithiasis, nephrocalcinosis or isolated proteinuria to more severe manifestations such as nephrotic syndrome (in some cases with a definite diagnosis of glomerulonephritis) or tubulopathy and interstitial nephritis. Hematological manifestations included autoimmune hemolytic anemia (25/96), thrombocytopenia (13/96) and neutropenia (6/96). Other conditions included autoimmune thyroiditis (15/96) and hepatitis (19/96), while food allergies (13/96), arthritis (8/96), alopecia (8/96) and lymphadenopathy (9/96) were occasionally reported during disease progression. Interestingly, among other uncommon manifestations (**Fig 1E**), neurological findings, of uncertain relation with *FOXP3* mutations, were reported in 16/96 patients, including: peripheral neuropathy, myopathies/hypotonia, hemidiaphragmatic paralysis, eosinophilic meningitis, neuro-developmental delay, seizures and benign intracranial hypertension.

The 96 patients included in the study displayed 33 published mutations^{4,5} and 21 novel mutations. The forkhead (FKH) domain emerges as a mutational hotspot of the *FOXP3* gene (**Fig 1F**), although mutations were scattered along the gene. Mutations in the N-terminal and FKH domains correlated with high variability of age at onset. The number of symptoms at onset was independent from the site of the mutations.

Laboratory findings

Table I summarizes the biological findings characterizing this IPEX patients' cohort before therapy. The distribution of lymphocyte subsets before IS or in patients treated with long-lasting IS or before HSCT was normal or seldom characterized by increased counts. The proportion of Treg cells, evaluated by flow cytometry, was available only in few patients and FOXP3 showed a wide range of expression. Gut biopsies frequently showed simultaneous involvement of several sites: the most common target was the small bowel, followed by large bowel, stomach and esophagus. The inflammatory infiltrates were usually polymorphic with predominance of lymphocytes and eosinophils. Villous atrophy remained the hallmark of IPEX enteropathy, although other nonspecific lesions associated with inflammatory damage (ulcers, crypt hyperplasia and abscesses) were present. Of note, 4 patients showed metaplastic lesions, either in the bowel or in the stomach, and one developed dysplastic lesions of the gastric mucosa. Anti-enterocyte, anti-harmonin (HAA) or anti-villin (VAA) autoantibodies were positive in 33/42 patients with enteropathy tested (**Table I**). In contrast, in 9/42 cases these autoantibodies could not be detected despite the presence of enteropathy, a finding possibly dependent on the choice of antibodies that were tested. Remarkably, in one case HAA positivity prompted the diagnosis in a patient with isolated IPEX-related gastritis. Anti-neuron antibodies were detected in one of the patients with neurological involvement. Finally, 12 patients screened positive for T1D-associated autoantibodies (2 of them had two positive autoantibodies), without having T1D (**Table I**). Thus, the histological and serological markers were indicative of the diagnosis only when considered together with the clinical manifestations.

Treatments

Of the 96 patients included in the study, 34 received IS and 58 underwent HSCT. Four patients, with *FOXP3* mutations either improved spontaneously (1), did not require IS (2), or was still asymptomatic (1), and were thus excluded from this analysis. One patient had several manifestations of the disease (enteropathy, growth retardation, eczema, autoimmune cytopenia) which improved spontaneously, with supportive therapy and without any immunosuppressive drugs. Other two patients had persistent signs of the disease which however did not require IS (eczema and growth retardation in one case and T1D in the other). The last patient is still asymptomatic at 6 years of age and he was diagnosed only due to familial history. **Figure 2** provides a schematic view of the disease evolution of each patient and his mutation, and reveals the variability of disease progression in our cohort.

Immunosuppressive therapy

The 34 patients who received IS and were not transplanted, started treatment at a median age of 1.5 years (range: 1 month - 19.6 years) with a median follow-up of 4 years (range: 1 month - 25 years). Only three patients had a follow-up shorter than 7 months.

Figure 3 shows the response to each drug, to drug combinations and the final outcome after IS. Twenty-five patients received systemic steroids (range: 2 months - 22 years, median 2 years). While steroid administration benefited 56% of the treated patients, it was often administered concomitantly with other immunosuppressive drugs (**Fig 3B**), thus direct effects cannot be confirmed.

Twenty patients received calcineurin inhibitors, either cyclosporine-A (CSA) or tacrolimus (FK506), with benefit in 40% (**Fig 3A**). Six of them also received concomitant steroids and 3 both steroids and non-calcineurin inhibitors. Among patients treated with non-calcineurin inhibitors (non-CNI), 15 patients received rapamycin and 19 received other immunosuppressive agents comprising azathioprine (AZA), methotrexate (MTX), mycophenolate-mofetil (MMF), mesalazine, sulfasalazine and 6-mercaptopurine. Combination of steroids with either calcineurin inhibitor, non-calcineurin inhibitors or both (12/21) infrequently (1/12) led to remission of autoimmunity (**Fig 3B**). Rapamycin improved autoimmune manifestations in 67% of patients (**Fig 3A**) and in 8/10 patients when it was used as monotherapy, with 6/8 patients achieving remission (**Fig 3B**). The use of AZA was beneficial in 36% of cases (**Fig 3A**). The use of monoclonal antibodies (mAbs), including anti-TNF α , anti-CD20 and, CTLA4 fusion protein (abatacept), was usually in addition to other IS therefore their efficacy as single drugs is not fully evaluable. Although treatment with a mAb was beneficial in the 52% of cases (**Fig 3A**), mAbs-based regimens rarely resulted in sustained remission of autoimmunity (2/8 patients) (**Fig 3B**).

Prior to initiation of IS, 25/34 patients were malnourished (74%) and 26/34 had intractable diarrhea (76%). The prevalence of these clinical manifestations significantly diminished following IS, since malnutrition persisted in 10 cases (29%; $p < 0.001$) while diarrhea persisted in 7 cases (20%; $p < 0.001$). Similarly, 3/5 patients with autoimmune hepatitis improved after IS. Nevertheless, prevalence of respiratory impairment, kidney dysfunction and frequency of infections increased under IS although not significantly ($p = 0.538$, $p = 0.511$, and $p = 0.627$) (**Fig 3C**).

Overall, 10/34 patients receiving IS completely controlled autoimmunity, while 24 patients still had autoimmune manifestations, mainly enteropathy (11/24). T1D remained the only autoimmune manifestation in 2 patients. In 11 patients new autoimmune manifestations arose while receiving IS, including T1D (4), thyroiditis (3), autoimmune cytopenia (2), autoimmune hepatitis (2), enteropathy (1), adrenal insufficiency (1), and arthritis (1). Overall, the OI score before starting IS, mostly corresponded to the OI score at disease onset, and did not significantly influence the outcome. Indeed, most of the patients improved in the short-term; whereas in the long-term the OI

score could worsen due to disease recurrence or progression and side effects of treatment, thus impacting survival (**Fig 3D**), as discussed below.

Nutritional support (enteral, parenteral or both) was often necessary for patients under IS (19/34), lasting for months or years. Recurrent hospitalization for disease complications and infections occurred a median of 4 times per year (ranging from 0 to 7 times per year). Thirty patients of 34 are alive at the last follow-up. Four patients died (at 5 months, 1.5, 7 and 22.5 years respectively). Causes of death were acute respiratory distress syndrome with multi organ failure, pneumonia, idiopathic cardiac arrest, and sepsis. The estimated overall survival of patients under IS was 86.8% (95% CI, 62.8 to 95.8) at 15 years and 65.1% (95% CI, 18.3 to 89.7) at 24 years (**Fig 5A**).

Thus, the merely symptomatic approach of long-term IS can be beneficial in the short term but did not prevent disease progression and development of complications in the majority of the patients and it could impact on patients' survival.

Hematopoietic stem cell transplantation

Fifty-eight patients underwent HSCT. Seven patients required a second transplant and one patient received three HSCTs. In these patients, follow-up records refer to the last procedure. The median follow-up was 2.7 years (range: 2 weeks - 15 years). The median age at onset for patients in this cohort was 1 month (range: birth - 1 year) and transplant was performed at a median age of 1.4 years (range: 1 month - 18.8 years), indicating that the majority of the transplants were performed at early age and close to disease onset (**Fig E1 A**). Despite the short timeframe between onset and transplant, 54 patients received IS prior to transplant. Disease manifestations improved before HSCT in 27/54 patients, while 23/54 patients had partial or no control of the autoimmune manifestations and 4/54 patients could not be assessed for response. Application of the OI score at the time of transplant demonstrated a strong association with outcome. The estimated overall survival after HSCT was 73.2% at 15 years, with significant differences for patients with either low or high OI score, as described below.

HSCT characteristics and complications (Table II). The majority of patients received reduced intensity transplant (RIT) (33/58) (see also **Table E1** in the Online Repository for further details about conditioning). Donor type included matched related (31/58), matched unrelated (21/58), haploidentical (5/58, 3 of which had alpha/beta T cell depletion) and other (1/58 mismatched related). Hematopoietic stem cell sources included bone marrow (35/58), peripheral blood stem cells (12/58) and cord blood (13/58). Anti-thymocyte globulin (ATG) or alemtuzumab (Alm) were frequently used (49/58 patients). GvHD prophylaxis mainly consisted of CSA associated with MMF or steroids. Hematological recovery was obtained at a median of 16 days for neutrophils and 20 days for platelets. At one year after HSCT, 22 of 33 patients had achieved T-cell reconstitution.

At a median of 14.5 months, 20/29 patients had positive proliferative response to mitogens. Independence from IVIG was reached at a median of 7 months in 30/45 patients. Transplant-related toxicity was observed in 11 cases (19%). Multiple infections were reported in 46 patients (85%).

GvHD. Nineteen (33%) of the transplanted patients experienced acute GvHD, which was of grade III-IV in 9. Among patients surviving more than 100 days, 6/52 developed chronic GvHD (10% of the transplanted patients). While not statistically significant, the incidence of acute GvHD was higher in patients who did not receive serotherapy (5/9; 55%) compared to those receiving ATG or Alm (16/47; 34%), for 2 patients GvHD could not be ascertained. Occurrence of acute GvHD was comparable ($p=0.2362$) in related (5/17; 29%) and unrelated (15/31; 48%) donor transplants. Overall, the incidence and severity of GvHD for IPEX syndrome were similar to that described for other primary immune deficiencies¹⁸.

Chimerism. Full donor peripheral blood chimerism was detected in 31/53 patients evaluated for chimerism; 17 of them were alive and in remission (or with T1D only). Among the patients with full donor chimerism, 3 died and 11 had autoimmune manifestations or GvHD). Mixed chimerism was detected in 18/53 patients and associated with disease remission in 9/18 (**Fig E1 B**). Importantly, the Treg cells were 100% of donor origin in 3/9 patients carrying mixed chimerism in remission. Moreover, 4/18 patients with mixed chimerism are alive with autoimmune manifestations (**Fig E1 C**) and 5/18 patients have died all but one of infections, at different times post-HSCT (**Fig E1 D**). The occurrence of mixed chimerism was not related to the use of RIT and was also observed following fully myeloablative conditioning. Overall, the data show similar proportion of patients in remission among those with full (17/31, 54%) or mixed (9/18, 50%) chimerism. In addition, graft failure was observed in 4/53 patients (**Fig E1 E**). Two patients experienced secondary graft failure between 3 and 6 months post-transplant, they are still alive but with disease relapse. The other two patients are dead, one with primary graft failure and the second one with acute graft loss forty days after transplant.

Survival after transplant. The estimated overall survival rate for transplanted patients at 15 years was 73.2%. The majority of deaths occurred in the first months after HSCT due to infections. Multivariable analysis showed that the type of conditioning, type of donor, and age at transplant did not significantly influence survival (**Fig 4A, B, C**). In fact, the pre-HSCT OI score was the only variable significantly influencing survival after HSCT. Indeed, the probability of survival was significantly lower in patients with a score between 3 and 5 as compared to patients with an initial score between 0 and 2 ($p=0.002$) (**Fig 4D**). In addition, the combined analysis of the score and of the conditioning regimen, showed that patients with score 0-2 had better survival independently of the administration of full conditioning or RIT. Within the same analysis, patients with score 3-5 who received full conditioning had better survival than the ones who received RIT. (**Fig 4E**).

Similarly, the OI score affected the survival outcome among patients below or above 1 year of age, with patients <1 year and high score performing significantly worse (**Fig 4F**). The majority of patients who were less than 1 year of age at the time of HSCT were also at or below the 3rd centile for weight (16/21 patients whose weight at transplant was reported). Among these patients, we further observed that the patients who did not survive had lower weight and younger age although the number of events was not sufficient to perform a multivariable analysis (**Fig E2 A, B**). DFS analysis showed that the probability of recurrent or new onset autoimmunity after transplant was not dependent on the conditioning administered (**Fig E2 C**) or chimerism obtained after transplant (**Fig E2 D**). Multivariable analysis did not identify a variable significantly affecting DFS after HSCT.

Comparison of outcomes between transplanted and not transplanted patients. Survival rates at 15 years among children undergoing HSCT were lower, although not significantly, as compared to those receiving chronic IS (73.2% vs 86.8%; $p=0.055$) (**Fig 5A**). This difference is largely due to the high mortality rate within 2.5 years following transplant, with up 15% of patients dying by the first 100 days and 25% by the first 2.5 years (**Fig 5A**). Patients who survive over 2.5 years after transplant do not show additional mortality up to 15 years later, and their probability of survival remains constant whereas that of IS patients, who have a longer follow-up, drops as the disease progresses and treatment complications increase with time (survival rate at 24 years 65.1%). Indeed, the survival of patients receiving IS does not depend on the OI score pre-IS but, in the long-term, a worsening of the OI score can negatively influence the survival in these patients. Thus, the OI score post-IS is significantly correlated with survival ($p=0.0444$) (**Fig 3D**).

The percentage of patients who completely resolved autoimmunity (or had only T1D) was higher in alive transplanted patients than in those receiving IS, although not significantly (56% vs 36%, $p=0.083$). Similarly, the persistence or the onset of new autoimmune manifestations (other than GvHD in transplanted patients) was significantly lower in patients surviving HSCT than under IS treatment (17% vs 51%, $p=0.001$) showing that IS does not prevent disease progression. This latter conclusion is also supported by the DFS curve showing a progressive reduction of DFS probability during IS. On the contrary, DFS probability remains stable after the first 6 years post-transplant (**Fig 5B**).

We did not observe any significant correlation between disease score or survival and the effect of the different mutations on the FOXP3 protein, as predicted by Polyphen-2 or PROVEAN, for patients given either IS or HSCT (**Fig E3**).

In the entire cohort from 28% (under IS) to 31% (after HSCT) of the patients experienced delayed neuro-motor development or needed a support teacher, despite comparable rates of patients undergoing schooling/working activities adequate for age after either therapeutic approaches. Of

note, a significant percentage of children needed psychological support. Nutritional issues lead to a frequent and prolonged use of feeding support in both categories. In line with the outcome results, a significant percentage of patients surviving HSCT, considered this therapy efficacious. In contrast, patients undergoing IS perceived an incomplete resolution of the disease and complained about chronic medications, side effects, and periodic follow-up (**Table E2**). This confirms that patients surviving transplant are less prone to disease evolution and complications over time.

DISCUSSION

The present retrospective study of IPEX patients provides an informative comparison of the currently available therapeutic options and their long-term outcome, together with a comprehensive and updated view of the disease and its initial presentation and progression. We demonstrate that IPEX patients have similar overall survival, regardless of whether they receive IS or HSCT, with a greater survival in the first years post-treatment for the non-HSCT group. However, the DFS of HSCT patients shows clear differences with stable resolution of autoimmunity as compared to the persistent disease progression in the non-transplanted IPEX patients. Therefore, the study highlights the therapeutic limitations of the current immunosuppressive regimens, although the use of rapamycin proved as the most beneficial IS therapy^{11,19,20} and appears to be superior to calcineurin inhibitors^{13,21-24}. Results from this study further indicate that a better survival outcome after HSCT is significantly affected by the patients' pre-HSCT conditions, as defined by the OI score we established. IPEX patients with low OI score, either initially or after IS, had a survival advantage after HSCT. On the contrary, IPEX patients with severe organ impairment (high OI score) at HSCT had the lowest chance of survival even receiving a RIT, suggesting that the clinical status is more important than the conditioning regimens in the outcome of HSCT. Other variables (i.e. type of donor, stem cells source, and chimerism) were not correlated with outcome, as previously reported in a small cohort of patients²⁵. In contrast though with this other cohort²⁵, our data show that patients transplanted before one year of age tended to have a lower survival (although without statistical significance), which may reflect either a more severe disease status or an increased sensitivity to conditioning agents. Overall, these data points to the value of optimizing patient's clinical condition prior to HSCT and considering HSCT before disease progression.

The majority of the patients in our study received a RIT²⁵⁻³⁰, conditioning that is not always associated with mixed chimerism, frequently observed in IPEX transplanted patients^{13,14,21,25,26,30}. Mixed rather than full donor chimerism did not affect DFS probability, as has been the case for other primary immunodeficiencies³¹. Importantly, in a small number of IPEX patients^{14,30} with mixed chimerism who achieved remission, all Treg cells were of donor origin, indicating that the presence of functional Treg cells may suffice to control autoimmunity, and supporting the importance of evaluating lineage specific chimerism.

In addition to the analysis of outcomes, results from the present data collection, strengthen the notion that the disease onset is usually early, with half of the patients presenting within the first month of life. We show that enteropathy, T1D and eczema are the initial symptoms, especially in neonates, while in the later onset patients, failure to thrive becomes a predominant symptom at presentation. With the increasing number of IPEX patients diagnosed overtime, a group of asymptomatic patients or patients initially presenting with only T1D have been identified. With

disease progression, multiple other autoimmune symptoms arise with blood, kidney and liver frequently targeted. Unexpectedly, neurological impairment, respiratory involvement and cardiac complications have been often observed, though their autoimmune origin rather than toxic or infectious pathogenesis remains unclear and should be investigated in a prospective study. As has been previously suggested^{9,32}, it is difficult to correlate the site of mutation with disease course or outcome. For example, mutations in the FKH domain, required for FOXP3 nuclear localization and DNA binding, correlated with the earliest onset, while there was no association between type of mutation and outcome. Further, the same genotype, can present with variable phenotypes. Indeed, asymptomatic children carrying the same mutation of their affected siblings have been recently published¹⁵ and herein reported (siblings carrying the c.1190 G>A mutation). Future prospective studies should be considered to better understand the functional effects of each *FOXP3* mutation, at disease onset and during disease course. Similarly, future studies should focus on evaluating the effect of different immunosuppressive regimens on the regulation of FOXP3 expression and its epigenetic modification such as demethylation at the Treg cell-specific demethylated region (TSDR), which is essential to maintain Treg cells identity^{33,34}. Overall, this retrospective study instructs that rapamycin should be the preferred choice as IS treatment, while HSCT should be considered in patients with low OI score and stable clinical conditions. In conclusion, our comprehensive description of the natural history of IPEX and comparison of the effects of IS and HSCT may assist in determining therapeutic choices for these complex patients.

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TABLE I. Biological findings characterizing the IPEX patients cohort before therapy.

Immunophenotype pre-IS	median; range (n. of patients tested)
Lymph tot /mmc	2925; 900-7480 (n=22)
CD3+ /mmc	2047; 600-4838 (n=23)
CD3+CD4+ /mmc	1276; 370-3658 (n=24)
CD3+CD8+ /mmc	586; 210-2020 (n=23)
RATIO CD4:CD8	2; 0.5-3.9 (n=23)
CD19+ /mmc	276; 0-1715 (n=22)
CD16+CD56+ /mmc	170; 20-525 (n=21)
CD4+CD25+ %	9; 0-35 (n=10)
CD4 ⁺ CD25 ⁺ CD127 ^{low} %	3; 1-6.6 (n=5)
FOXP3+ % in CD4+CD25+CD127 ^{low}	36; 2,2-58 (n=5)
FOXP3+ % in CD4+	5; 0,8-7 (n=4)
Immunophenotype pre-HSCT	median; range (n. of patients tested)
Lymph tot /mmc	3350; 440-8189 (n=39)
CD3+ /mmc	2185; 350-9388 (n=52)
CD3+CD4+ /mmc	1311; 150-7824 (n=52)
CD3+CD8+ /mmc	781; 106-2612 (n=51)
RATIO CD4:CD8	1.9; 0.3-10.1 (n=51)
CD19+ /mmc	572; 0-6245 (n=51)
CD16+CD56+ /mmc	200; 0-1112 (n=45)
CD4+CD25+ %	13; 0-49 (n=18)
CD4 ⁺ CD25 ⁺ CD127 ^{low} %	7.8; 3.3-20.2 (n=4)
FOXP3+ % in CD4+CD25+CD127 ^{low}	50; 1.3-98 (n=9)
FOXP3+ % in CD4+	0; 0-5 (n=3)
Gut biopsies before therapy (tot. n=61)	n. of cases
<i>Number of sites involved</i>	
single	26
multiple	34
<i>Affected sites</i>	
0 esophagus	7
1 stomach	16
2 small bowel	45
3 large bowel	16
4 all sites	7
<i>Combinations of affected sites</i>	
small and large bowel	11
stomach + small bowel	9
stomach + large bowel	1
esophagus + stomach	1
esophagus + small bowel	2
stomach + small and large bowel	2
esophagus + stomach + small bowel	1
esophagus + small and large bowel	1
<i>Infiltrating Cell Type</i>	
1 eosinophils	19
2 lymphocytes	32
3 plasmacells	8
4 polymorphonucleated cells	6
5 polymorphic infiltrate including all cells	8
6 unspecified inflammatory cell type	4
<i>Histological lesions</i>	
villous atrophy	42
loss of goblet cells	3
crypt hyperplasia	9
crypt abscesses	8
Ulcers	9
loss of parietal cells	2
Metaplasia	4
Enteropathy-related autoantibodies	n. positive / n. of cases tested
n. pts positive for anti-enterocyte Abs	25/34
n. pts positive for HAA	13/24
n. pts positive for anti-enterocyte and HAA	4/5
n. pts positive for VAA	1/12
Other autoantibodies before therapy	n. positive / n. of cases tested
Tot. n. of patients tested for GAD	41
n- pts positive for GAD among pts with T1D	15/18
n- pts positive for GAD among pts without T1D	9/23
Tot. n. of patients tested for IA	27
n- pts positive for IA among pts with T1D	9/15
n- pts positive for IA among pts without T1D	0/12
Tot. n. of patients tested for IAA	33
n- pts positive for IAA among pts with T1D	10/13
n- pts positive for IAA among pts without T1D	3/20
Tot. n. of patients tested for ZNT8	14
n- pts positive for ZNT8 among pts with T1D	1/8
n- pts positive for ZNT8 among pts without T1D	0/6
Tot. n. of patients tested for ICA	6
n- pts positive for ICA among pts with T1D	1/3
n- pts positive for ICA among pts without T1D	3/3

Abbreviations: GAD, Glutamic Acid Decarboxylase Autoantibodies; HAA, anti-harmonin autoantibodies; IA, islet antigen; IAA, anti.insulin autoantibodies; ICA, anti-islet cell antibodies; VAA anti-villin autoantibodies; ZNT8, zinc transporter 8 autoantibodies

TABLE II. Characteristics, outcomes and complications of HSCT

	n. patients	%
Characteristics		
Total n. of patients	58	
Age (y) at transplant: median (range)	1.4 (0.2-18.8)	
N of patients who received multiple transplants		
2 HSCT	7	
3 HSCT	1	
Conditioning		
Full	27	47
RIT	31	53
Donor - related ; unrelated		
Matched	10 ; 21	17 ; 36
1 MM	1 ; 13	2 ; 22
2 MM	0 ; 6	0 ; 10
3 MM	1 ; 1	2 ; 2
Haplo	5 ; 0	9 ; 0
HSC source		
BM	35	60
PB	11	19
CB	12	21
Cell doses		
BM (TNC*10 ⁸ /kg; median; range)	7.1 (0.01-91.3)	
PB (CD34*10 ⁶ /kg; median; range)	11.4 (4.3-40)	
CB (TNC*10 ⁷ /kg; median; range)	9.8 (0.6-42)	
Serotherapy		
ATG	22	38
Alm	27	46
None	9	16
GvHD prophylaxis		
CSA + MMF	18	31
CSA + steroids	10	17
CSA	6	10
MTX + CSA (with or w/o short course of steroids)	8	14
MTX + FK506 + steroids	7	12
Others	9	16
Bone marrow recovery		
neutrophils (days after HSCT: median; range)	16 (3-33)	
platelets (days after HSCT: median; range)	20 (5-114)	
Immunoreconstitution		
n. patients with T cells > 1000/mm ³ at 1 y	22 out of 33	
positive PHA response	14.5 (3-60)	
(months after HSCT: median; range)		
independence from IVIg substitution	7 (1-48)	
(months after HSCT: median; range)		
Use of donor stem cell boost	3	
Use of donor lymphocytes infusion	3	
Complications		
Transplant-related toxicity	11	20
Infections	46	79
Graft-versus-host disease (total)	21	36
aGvHD (grade I-IV)	19	33
aGvHD (grade III-IV)	9	16
cGvHD	6	10
no GvHD	37	64
Deaths	15	26

*Toxicity after HSCT consisted of mucositis, pneumonitis, posterior reversible encephalopathy, undefined hypertrophic cardiomyopathy, nephropathy and hepatic sinusoidal obstruction syndrome.

Abbreviations: Alm, alemtuzumab; aGvHD, acute graft versus host disease; ATG, anti-thymocyte globulin; BM, bone marrow; CB, cord blood; cGvHD, chronic graft versus host disease; CSA, cyclosporine; FK506, tacrolimus; Full, full conditioning regimen; MM, mismatch; MMF, mycophenolate mofetil; MTX, methotrexate; PB, peripheral blood; PHA, phytohemagglutinine; RIT, reduced intensity transplant; TNC, total nucleated cells.

TABLE E1. Conditioning regimens.

Conditioning		FULL (tot 29)	RIT (tot 29)
Bu + Flu	+ Alm	2	3
	+ ATG	1	4
Bu + Cy	+ Alm	3	-
	+ ATG	4	-
	\	2	-
Treo + Flu	+ Alm	1	4
	+ ATG	-	2
Mel + Flu	+ Alm	-	8
	+ ATG	-	2
Treo + Flu + Thio	+ Alm	2	-
	+ ATG	3	-
Bu + Flu + Cy ($\pm$ TBI)	+ ATG	6	-
Others		5	6

Abbreviations: Alm, alemtuzumab; ATG, anti-thymocyte globulin; Bu, busulfan; Cy, cyclophosphamide; Flu, fludarabine; Full, full conditioning; Mel, melphalan; RIT, reduced intensity transplant; TBI, total body irradiation; Thio, thiotepa; Treo, treosulfan.

TABLE E2. Summary quality of life (QoL) questionnaire.

	HSCT (n=42)	IS (n=26)	p value
Age - years * (median; range)	10 (2-24.4)	13.25 (0.3-28.8)	
QUESTION			
Q1. Adequate age schooling/working activity	30/38 (79)	18/23 (78)	ns
Q2. Regular schooling/working activity	28/35 (80)	16/23 (69)	ns
Q3. Abnormal neuromotor development	11/39 (28)	8/25 (32)	ns
Q4. Support teacher	9/34 (26)	8/23 (35)	ns
Q5. Normal nutrition	33/41 (80)	16/25 (64)	ns
Q6. Normal relationships	33/38 (87)	16/22 (73)	ns
Q7. Psychological support	7/37 (19)	6/24 (25)	ns
Q8. Considers the therapy efficacious	33/36 (92)	15/22 (68)	0.052
Q9. Complains about medicines	2/34 (6)	12/33 (36)	<0.001
Q10. Complains about side effects	2/35 (6)	8/23 (35)	0.012
Q11. Complains about follow-up	4/36 (11)	11/22 (50)	0.002

FIGURE LEGENDS

FIG 1. Patients' demographics and disease-related characteristics. **A)** Diagnostic delay scatter plot displaying correlation between age at diagnosis and age at onset (n=96). Spearman's rank correlation $\rho=0.456$, $p<0.001$. **B)** Histogram distribution of number of patients diagnosed between 2001-2015. Overlaid density distribution showed in blue. **C)** Heat map of number symptoms at onset grouped by age at onset. Symptoms present in each age group (indicated by rows) were scaled (z-score or standardized score) and then converted to colors from yellow (low=less frequent) to red (high=highly frequent). NA=3, one patient has the mutation but has not yet experienced the onset, and for two patients the first symptom is unknown. **D)** Bar graph comparing frequency of symptoms at onset and later during disease evolution. Every bar indicates number of patients presenting each symptom. However, each patient can exhibit more than one symptom at once. Abbreviations: AT, autoimmune thyroiditis; FTT, failure to thrive; AIN, autoimmune neutropenia; AIHA, autoimmune hemolytic anemia; ITP, idiopathic thrombocytopenic purpura; Neph, nephropathy; LN, lymphadenopathy). **E)** Uncommon manifestations. Every bar indicates number of patients presenting each symptom. **F)** Scatter plot of *FOXP3* gene mutations grouped by domain, indicating age at onset and number of symptoms at onset, with median (n=87, for 10 patients cDNA *FOXP3* mutations were not specified). Gene structure: N-terminal proline-rich (PRR) domain (orange), zinc-finger (ZF) domain (green), leucine-zipper (LZ) domain (blue), LZ-forkhead (FKH) loop (yellow), and FKH domain (red). Mutations were grouped as follows: <c.1 to c.570> = E1-5 N-terminal domain, <c.591 to c.666> = E5-6 ZF domain, <c.717 to c.780> = E6-7 LZ domain, <c.781 to c.1010> = E7-9 LZ-FKH loop, <c.1011 to c.1251> = E9-11 FKH domain.

FIG 2. Timeline of natural history and disease evolution. **A)** Patients undergoing HSCT and **B)** patients undergoing IS. Each line represents a patient identified by his *FOXP3* mutation in order of localization on the gene. The end on the line represents the last day of follow up, and different symbols represent age at onset and age at HSCT or the beginning of IS. An "X" at the end of the line indicates the age of death. The color of the line indicates the disease status after treatment, either if the patient went into remission (blue), still diabetic (grey) or not cured (red). NA=not available.

FIG 3. Immunosuppressive therapy. **A)** Bar graph indicating numbers of patients exhibiting response, partial benefit or no response according to a specific immunosuppressive drug administered (n= 34, however each patient received more than one drug). **B)** Bar graph indicating each patient's outcome after treatment with a combination of drugs, as last treatment. Each bar represents the number of patients receiving the treatment, distinguishing those in remission from those with additional or persistent autoimmunity (n=34). **C)** Pre- and post- IS patients' conditions, each bar represents the number of patients presenting each condition. On the right side the relative percentage is reported (n=34, however each patient could present more than one condition at once). Abbreviations: Ca Inhib, calcineurin inhibitors; RAPA, rapamycin; AZA, azathioprine; MTX, methotrexate; MMF, mycophenolate mofetil; Non-Ca Inhib, Non-calcineurin inhibitors; mAbs, monoclonal antibodies; "others", any different IS (for example: 6-mercaptopurina, mesalazine...). **D)** Percent of survival of patients undergoing IS (n=34) according to score post-IS ($p=0.0444$).

FIG 4. Survival analysis of patients undergoing HSCT. Percent of survival of patients undergoing HSCT (n=58) according to **A)** conditioning (Log-rank test, $p=0.234$), **B)** donor type ($p=0.886$), **C)** age at HSCT ($p=0.359$), **D)** score pre-HSCT ($p=0.003$), **E)** score and conditioning ($p=0.010$) and **F)** score and age at HSCT ($p=0.019$). A survival probability table accompanies those plots that show significant differences (time points: 6 months, 1,3,5, and 10 years).

FIG 5. Probability of survival and disease status after treatment. **A)** Survival analysis of IPEX patients undergoing HSCT or IS (n=92, $p=0.055$) **B)** Disease free survival analysis of IPEX patients undergoing IS or HSCT censored for deaths (n=81, $p= 0.419$).

SUPPLEMENTARY FIGURES

FIG E1. Age distribution and chimerism. A) Histogram distribution of number of patients undergoing HSCT from 0 to 20 years grouped by age at onset. Percentage of donor chimerism at different time points in patients who experienced B) mixed chimerism with remission, C) mixed chimerism with persistence of autoimmunity, D) death, and E) graft failure.

FIG E2. Influence of age, weight, conditioning, chimerism on HSCT outcome. Association of survival with score and A) age at HSCT (n=25) or B) weight at HSCT (n=21) among patients undergoing HSCT at 1 year of age or below. Bars show mean with SD. Disease free survival curve, of patients who underwent HSCT according to C) type of conditioning (n=50, p= 0.712) and D) chimerism (n=44, p= 0.899). Patients who died early after transplant or without any autoimmunity were excluded from this analysis.

FIG E3. Predicted mutation effect on protein structure and function. Summary statistics of protein effect score predicted by PROVEAN protein web tool (score: -30-0, deleterious to neutral) between A) alive and dead patients, and B) among patients with different scores pre-HSCT. Summary statistics of protein effect score predicted by PolyPhen-2 algorithm (score: 0-1, benign to damaging) between C) alive and dead patients, and D) among patients with different scores pre-HSCT. No significant differences were found between the groups analyzed. Each point represents a patient, the column height indicates the median score, and the error bars represent the standard deviation.

Figure 1

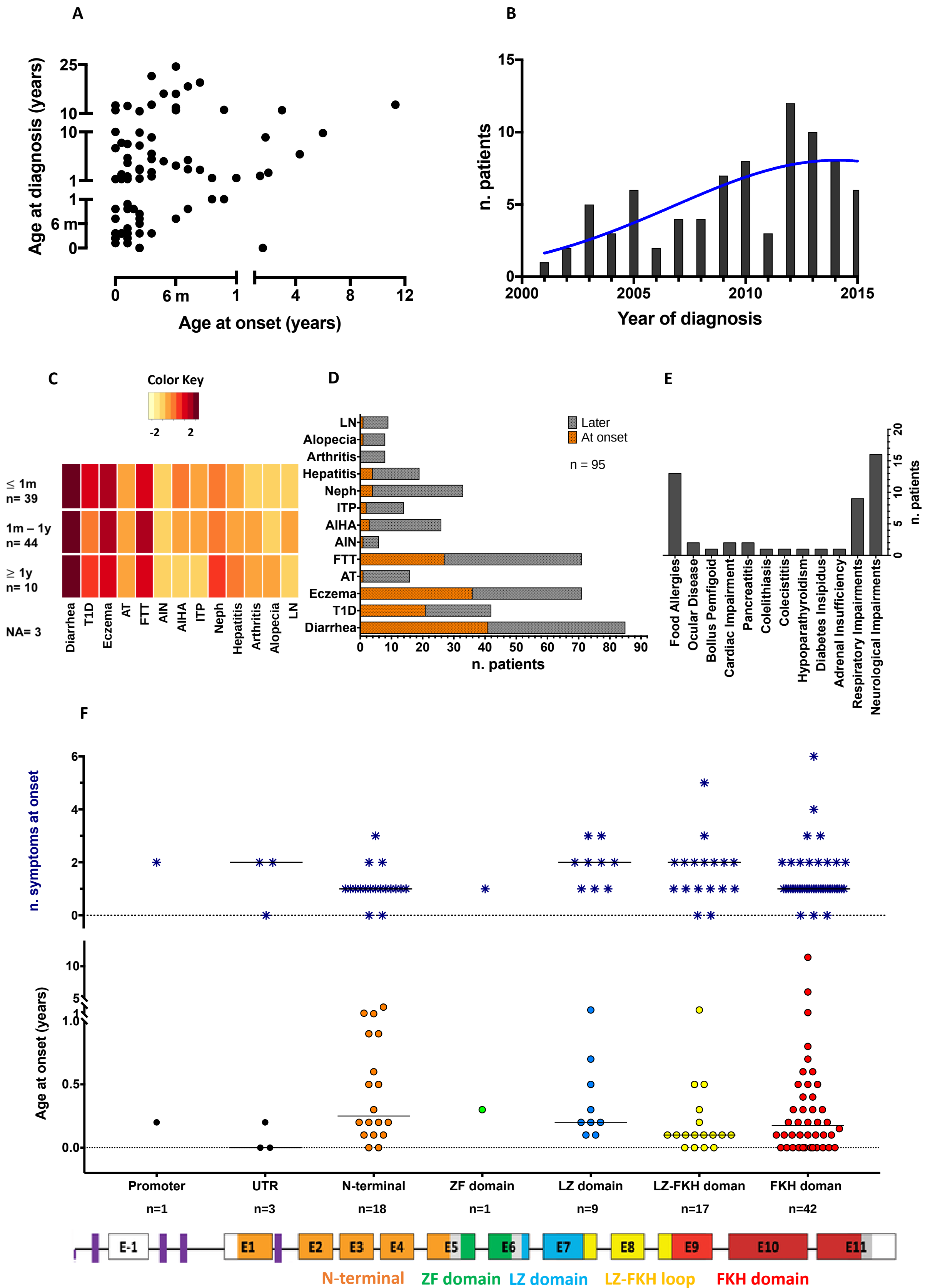
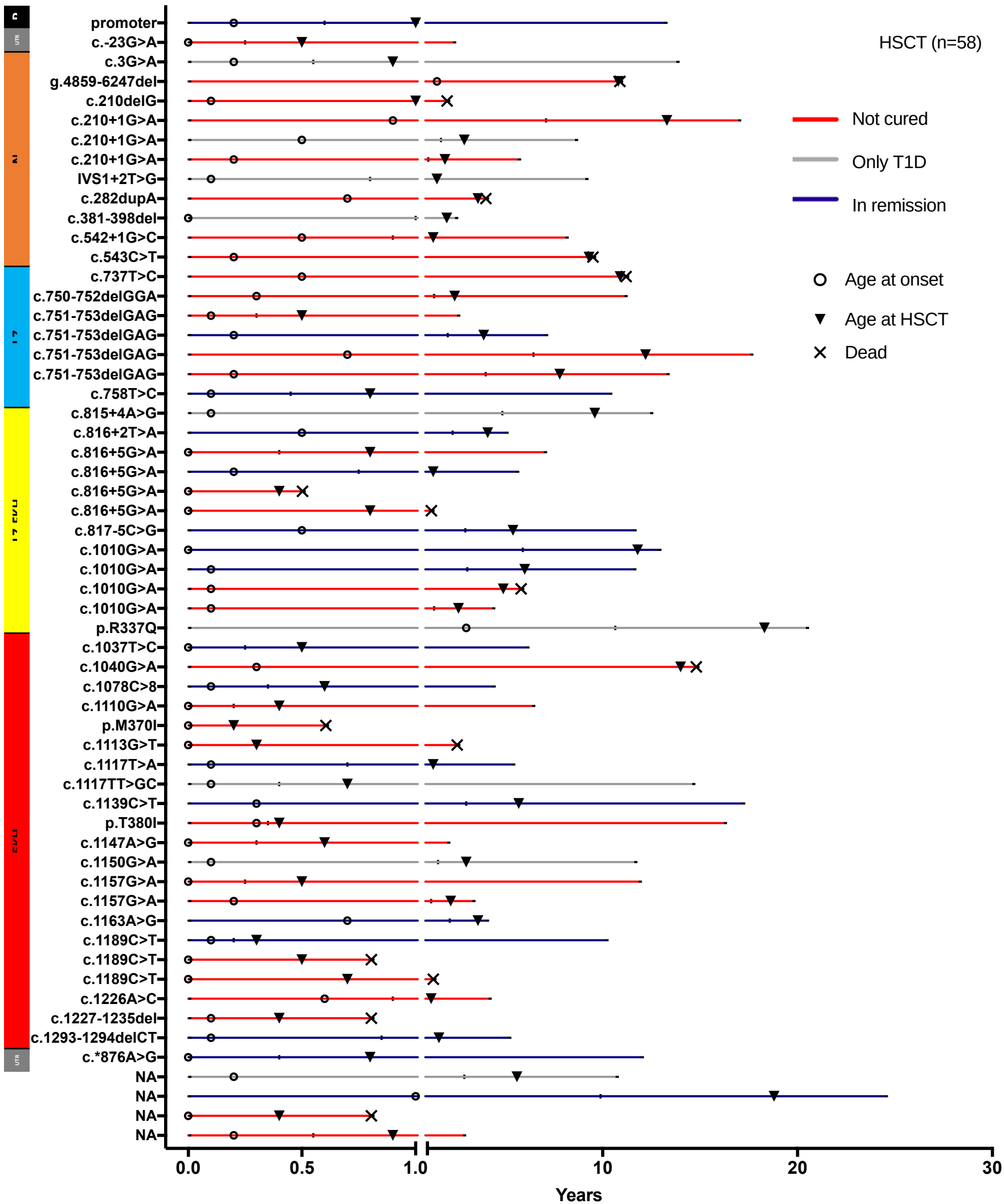


Figure 2

A



B

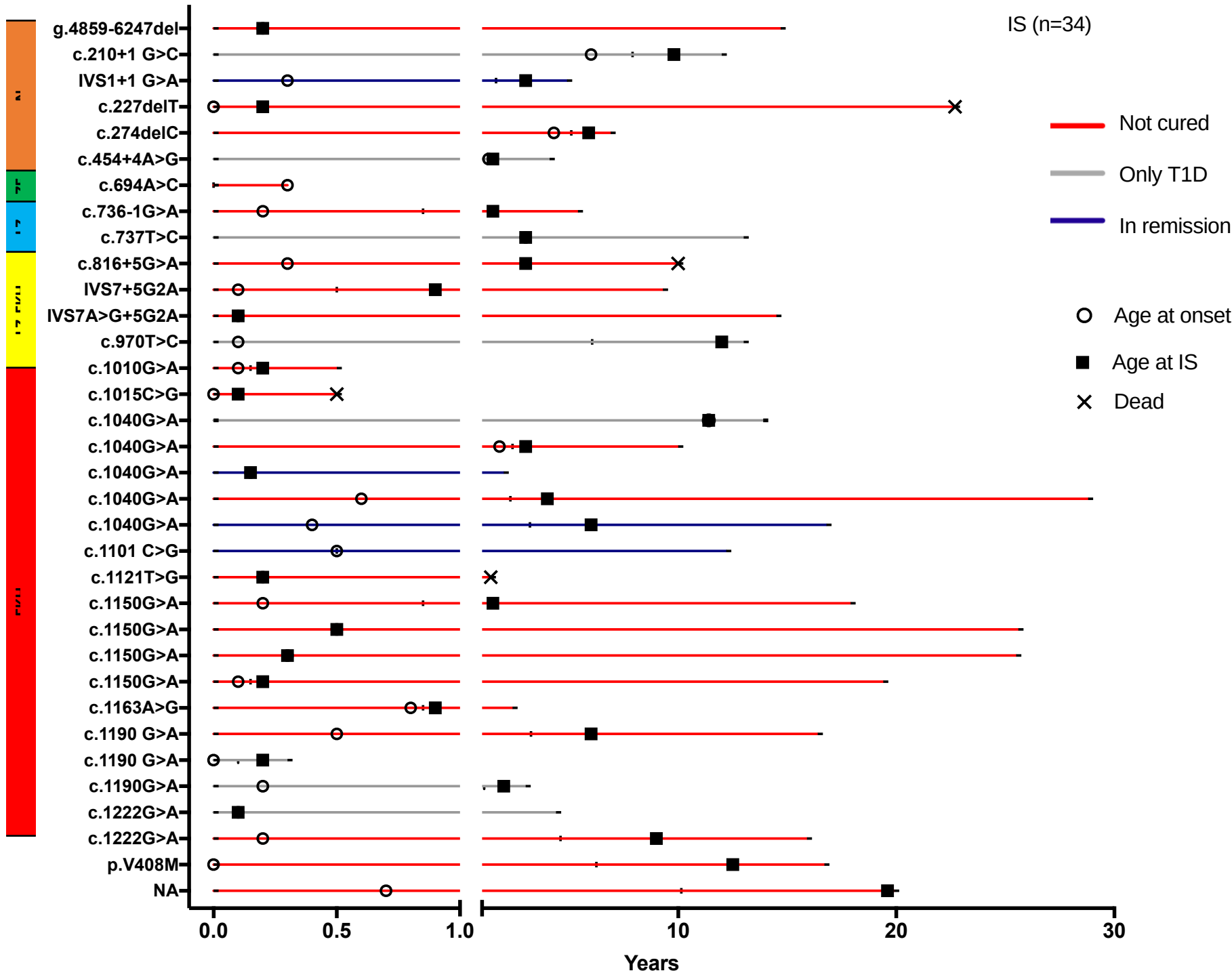


Figure 3

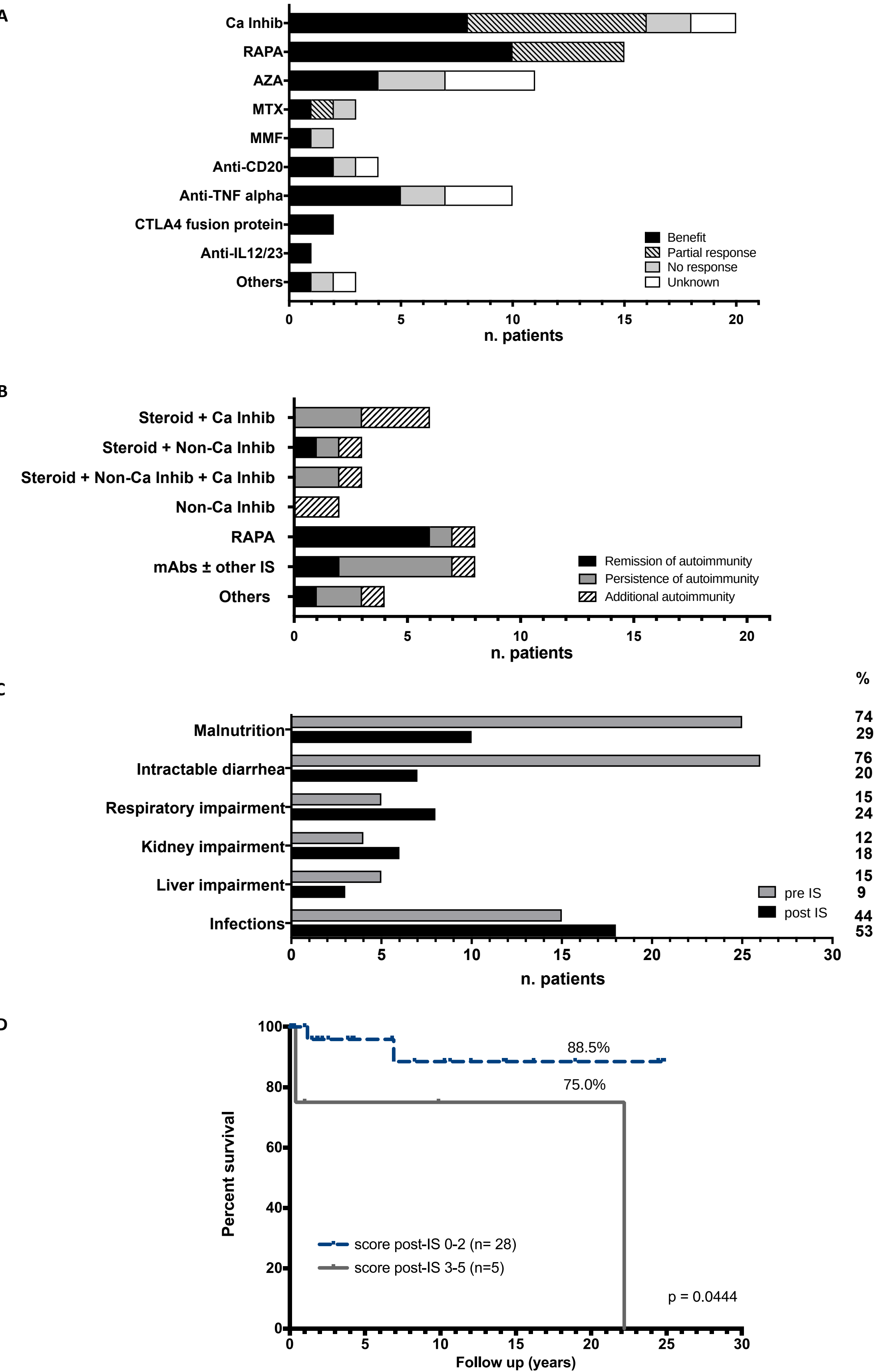
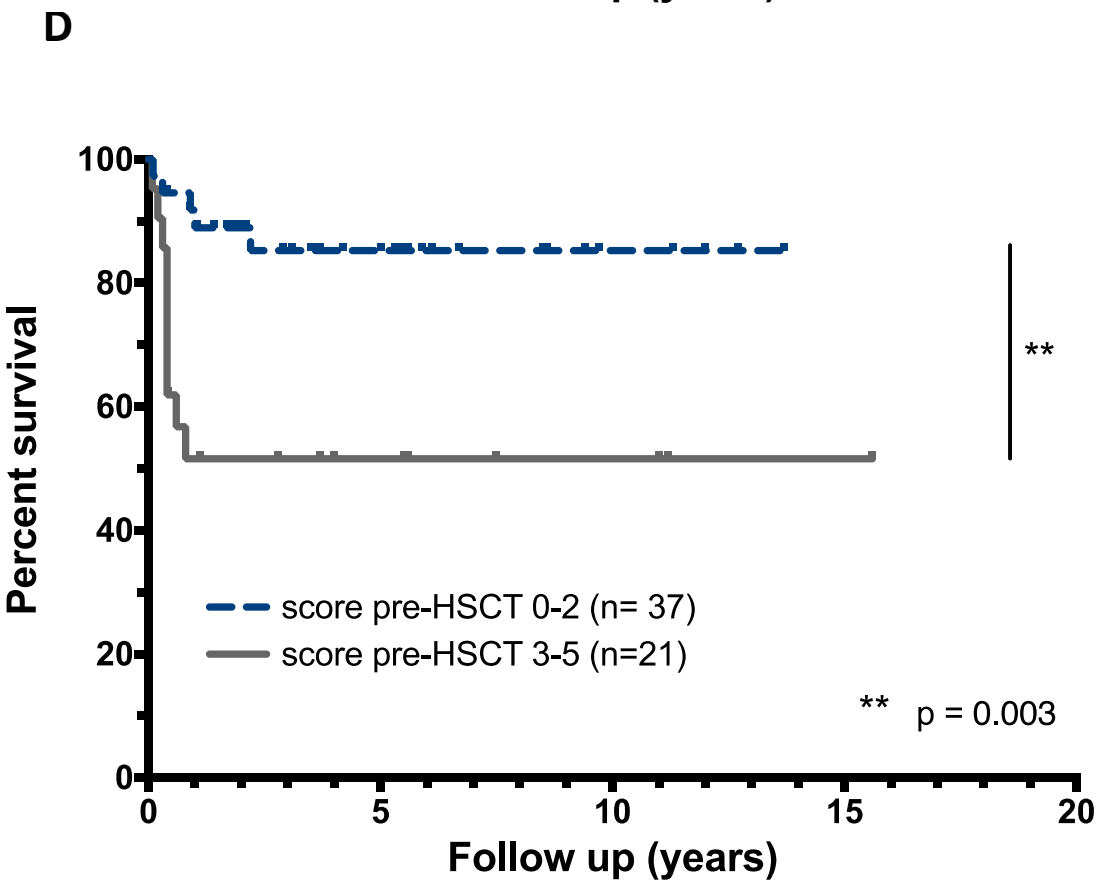
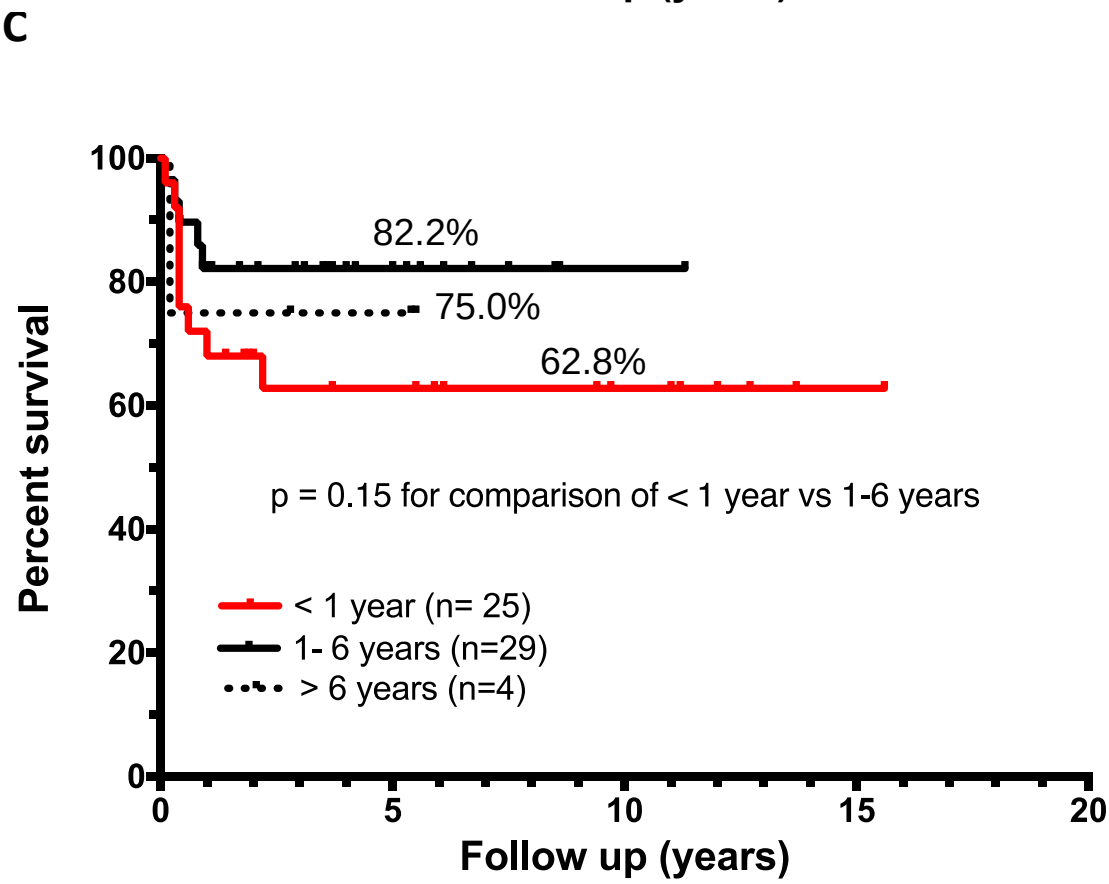
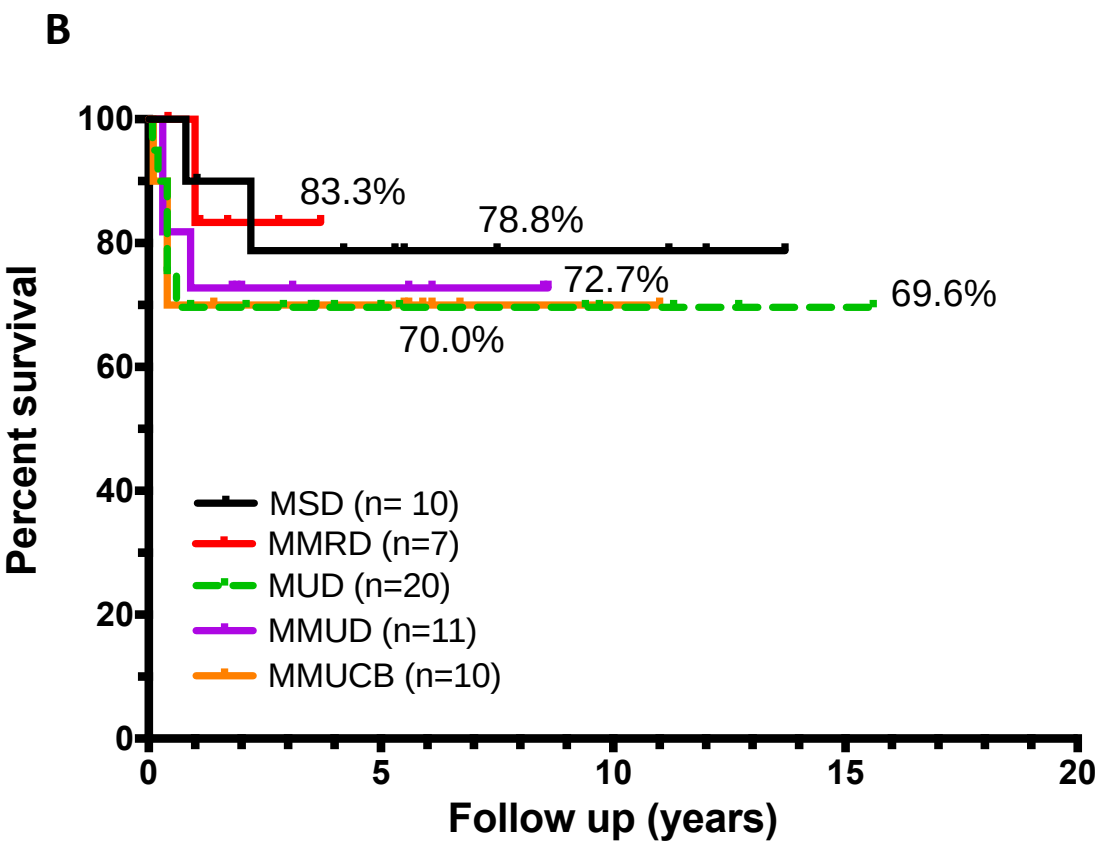
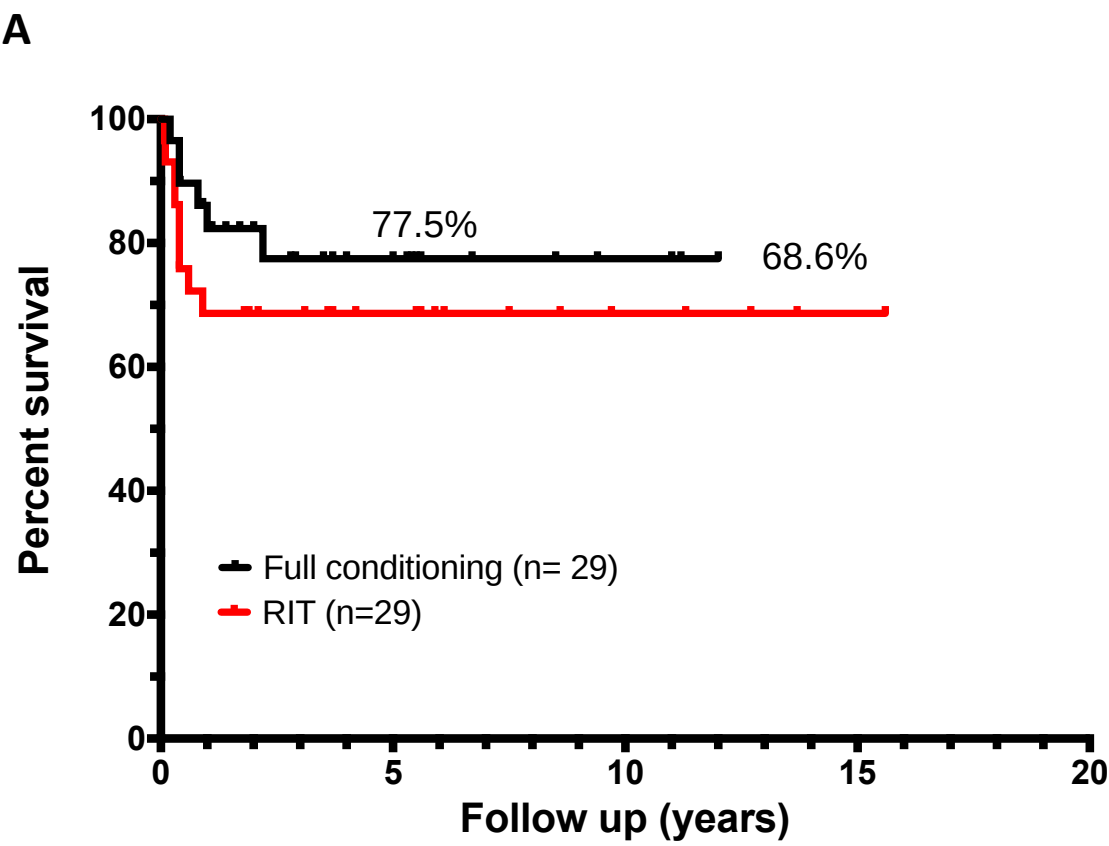
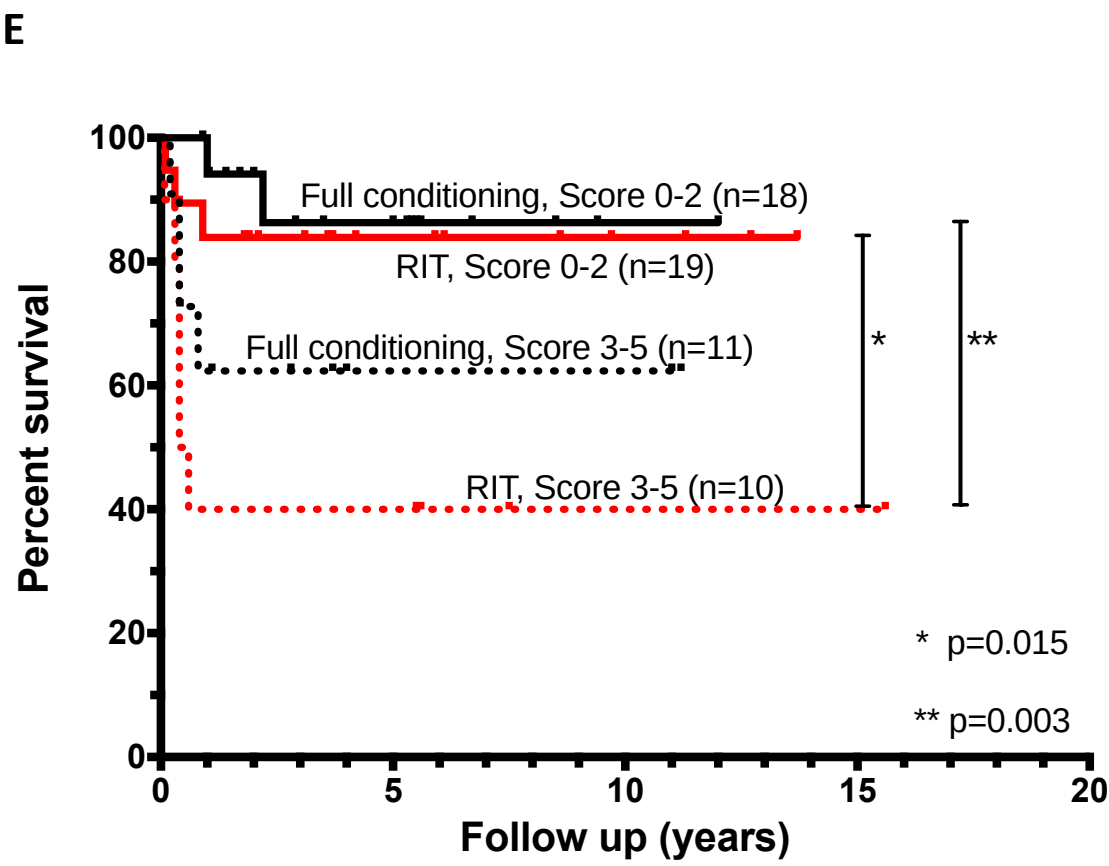


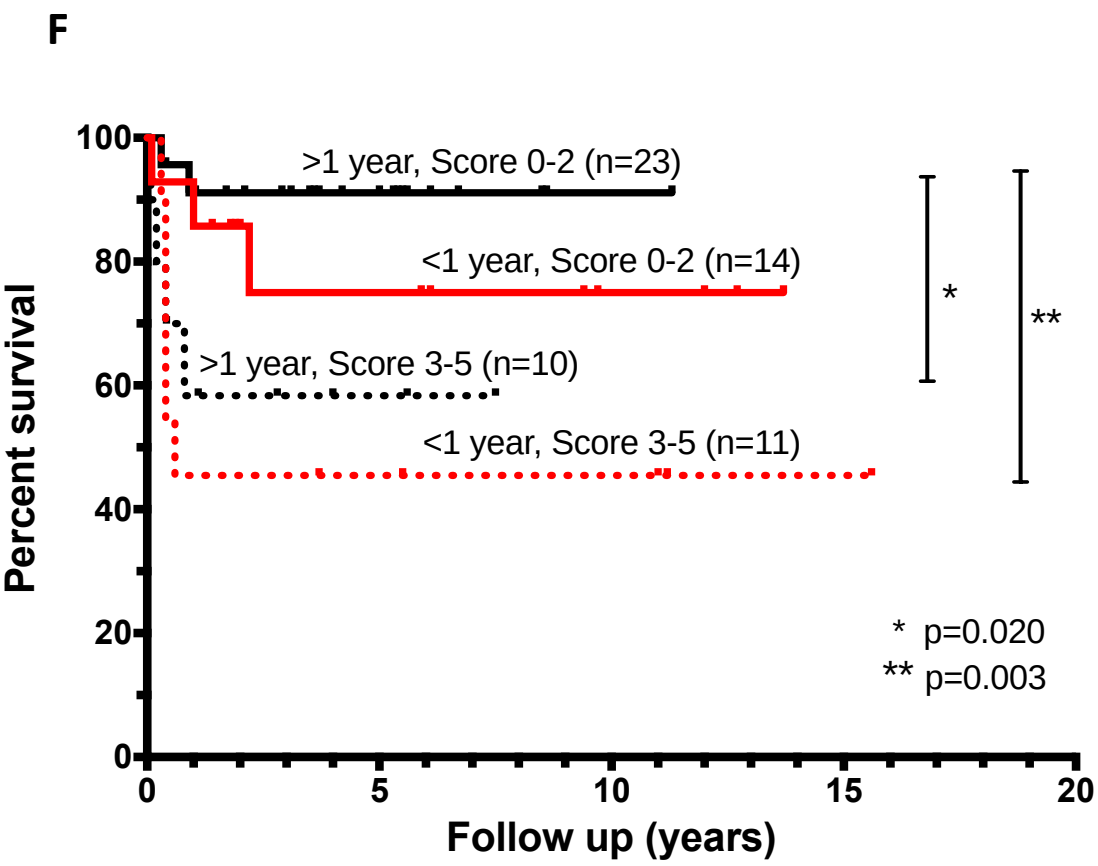
Figure 4



Score	Survival probability (%)				
	6m	1y	3y	5y	10y
Score 0-2	94.6	88.9	88.9	85.2	85.2
Score 3-5	61.9	51.6	51.6	51.6	51.6

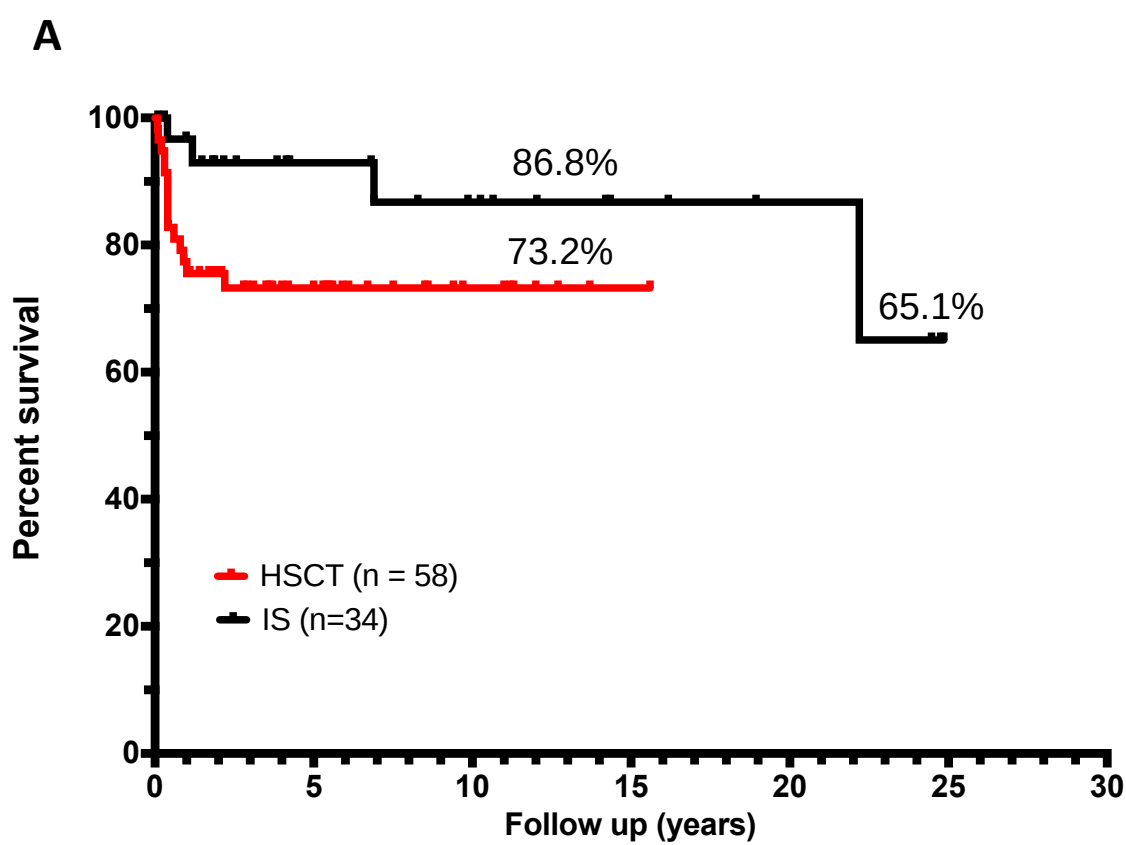


Full C, Score	Survival probability (%)				
	6m	1y	3y	5y	10y
Full C, Score 0-2	100.0	94.1	86.3	86.3	86.3
RIT, Score 0-2	89.5	83.9	83.9	83.9	83.9
Full C, Score 3-5	72.7	62.3	62.3	62.3	62.3
RIT, Score 3-5	50.0	40.0	40.0	40.0	40.0

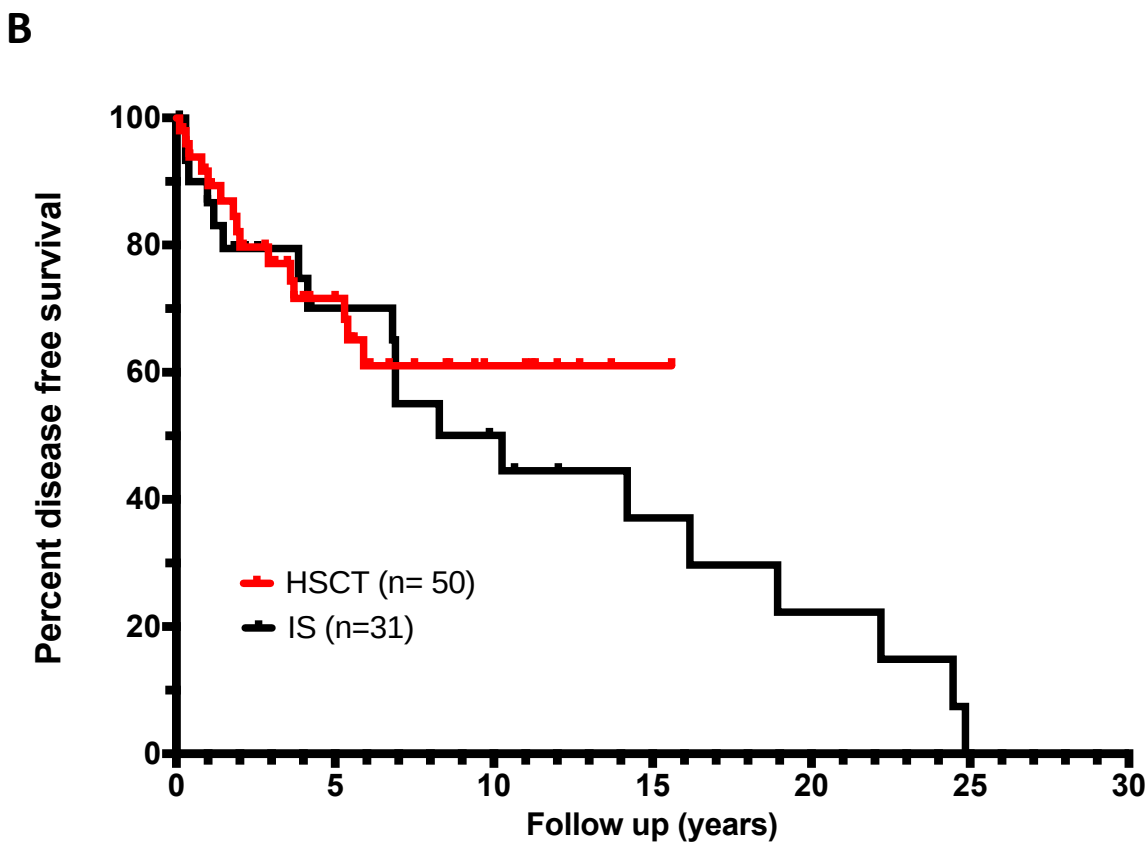


Time	Survival probability (%)				
	6m	1y	3y	5y	10y
>1 year, Score 0-2	95.7	91.1	91.1	91.1	91.1
<1 year, Score 0-2	92.9	85.7	85.7	75.0	75.0
>1 year, Score 3-5	70.0	58.3	58.3	58.3	58.3
<1 year, Score 3-5	54.6	45.5	45.5	45.5	45.5

Figure 5



	Survival probability (%)				
	6m	1y	5y	10y	15y
HSCT	82.8	75.5	73.2	73.2	73.2
IS	96.7	96.7	92.9	86.8	86.8



	Survival probability (%)				
	6m	1y	5y	10y	15y
HSCT	93.9	89.3	71.6	61.0	61.0
IS	90.0	83.1	65.1	50.1	37.1

Figure E1

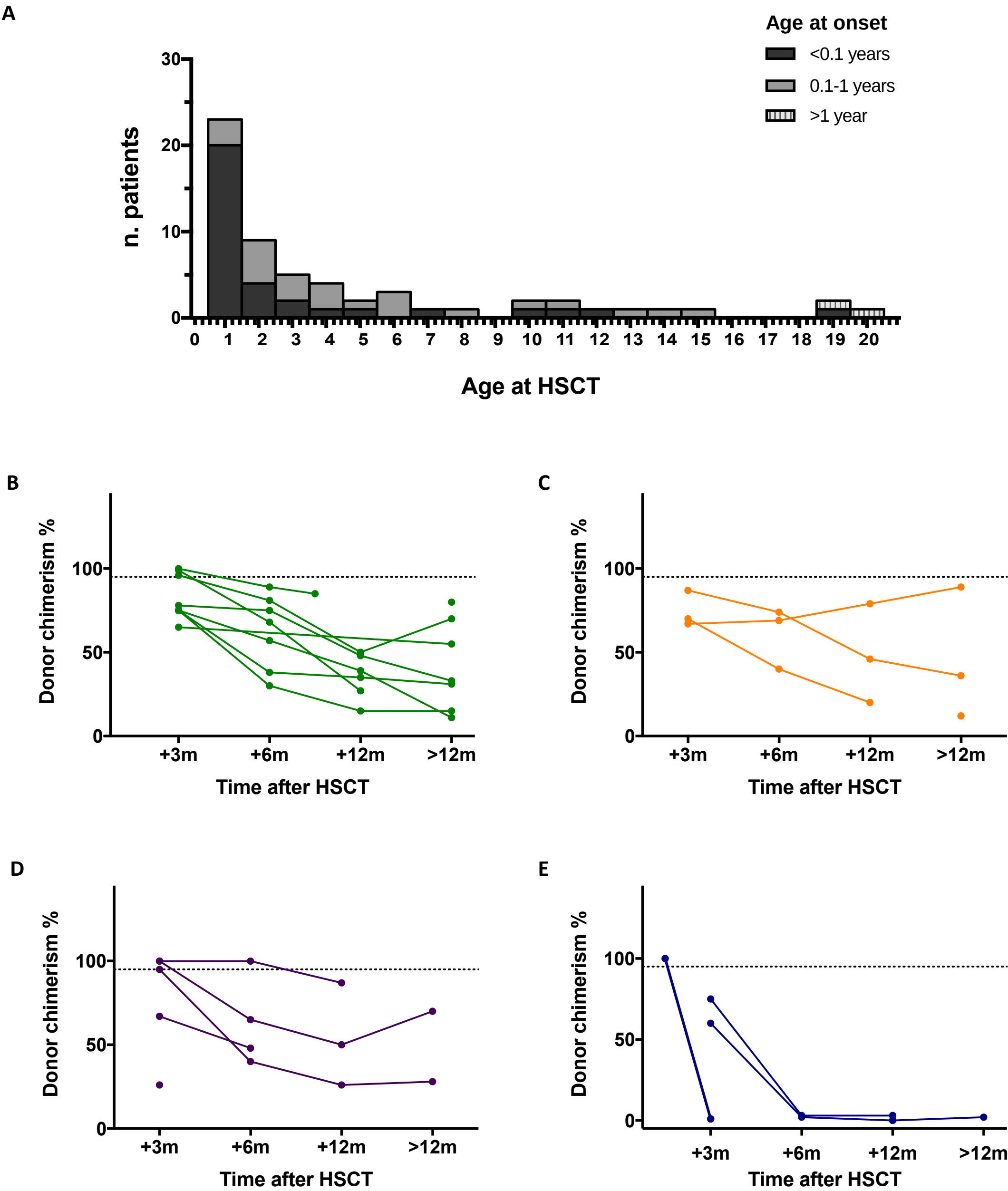


Figure E2

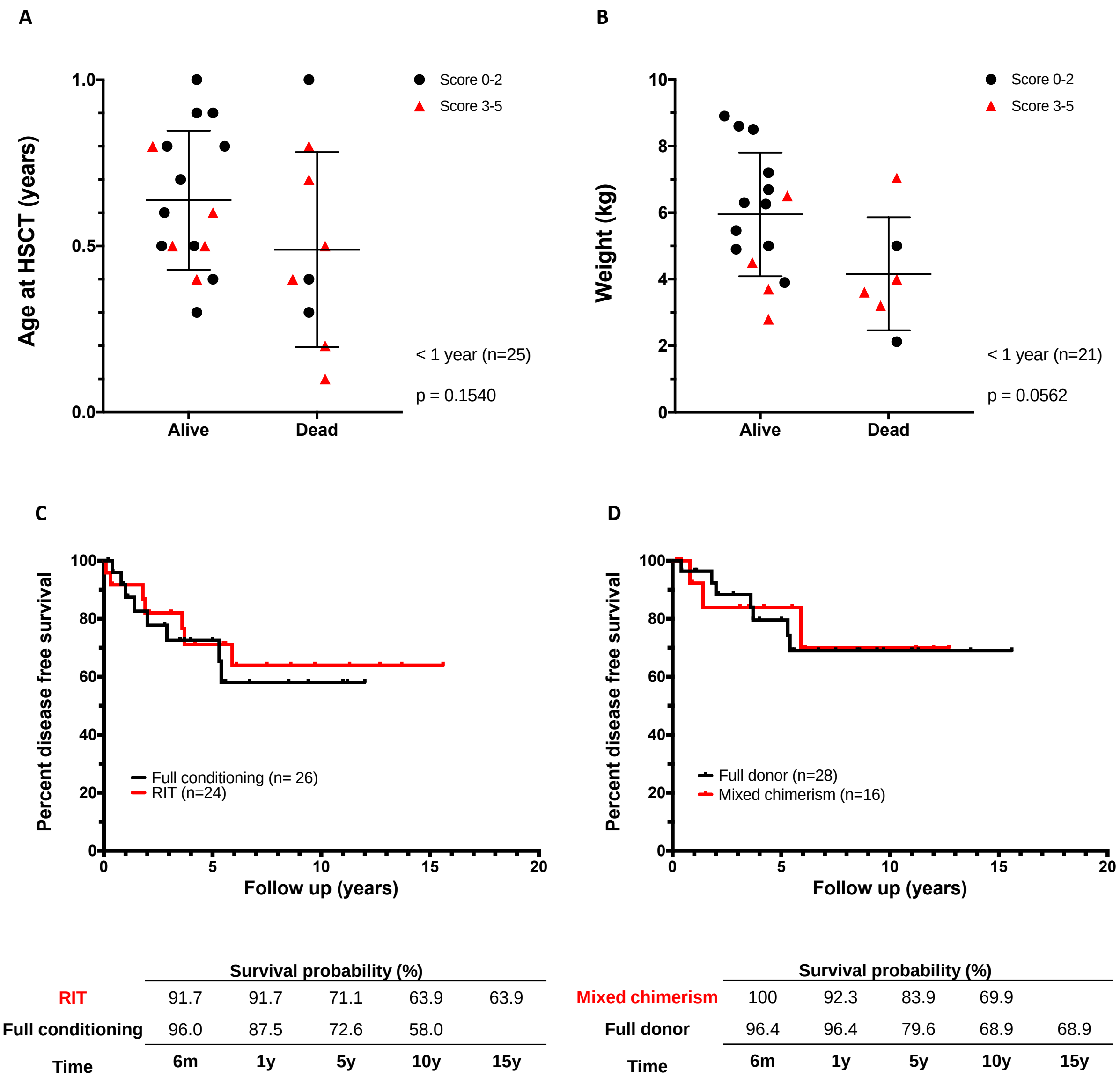
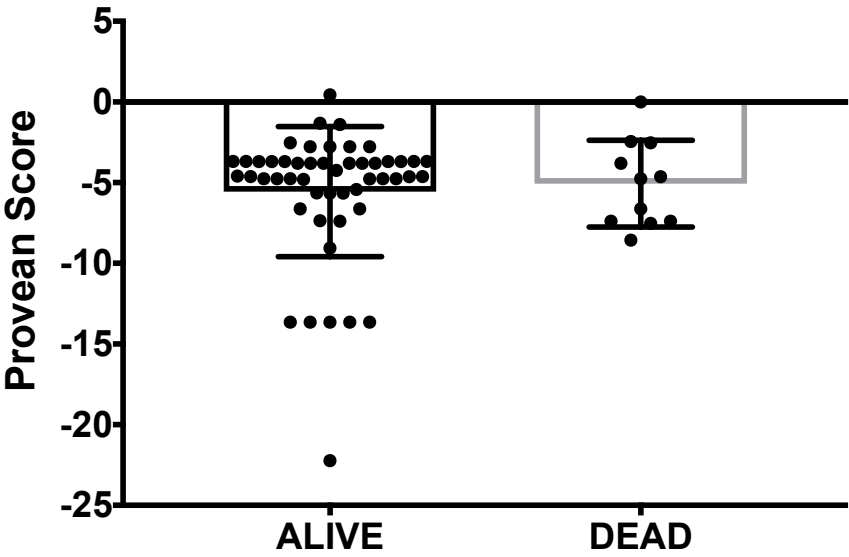
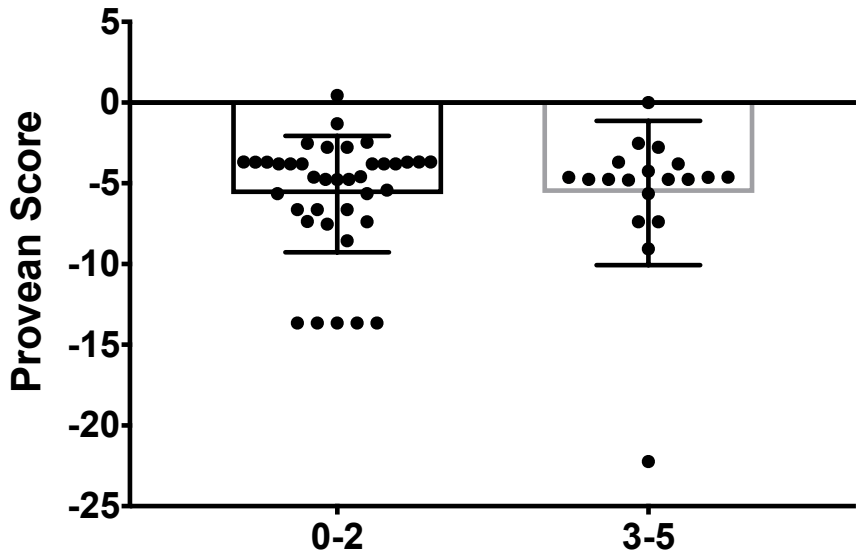


Figure E3

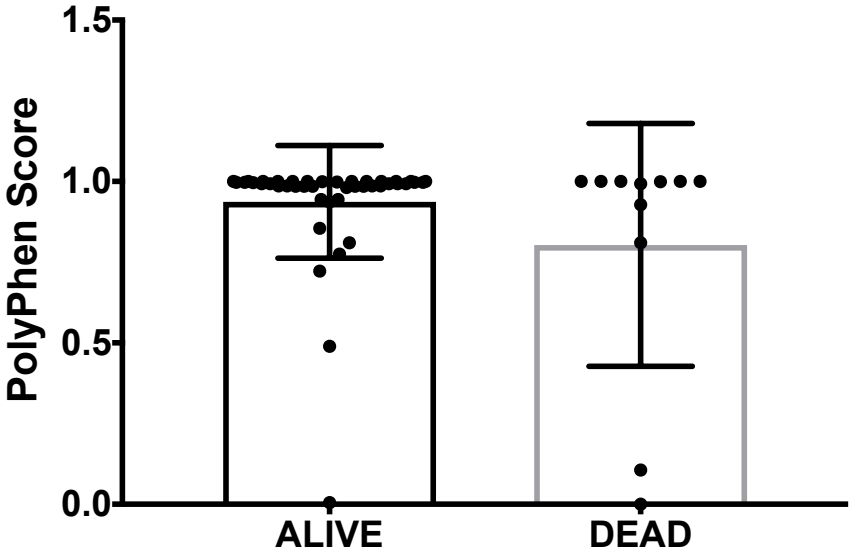
A



B



C



D

