

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Association between Thymic Function and Allogeneic Hematopoietic Stem Cell Transplantation Outcome: Results of a Pediatric Study

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1508685> since 2020-02-26T12:06:46Z

Published version:

DOI:10.1016/j.bbmt.2015.02.010

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1 **ASSOCIATION BETWEEN THYMIC FUNCTION AND ALLOGENEIC**
2 **HEMATOPOIETIC STEM CELL TRANSPLANTATION OUTCOME: RESULTS**
3 **OF A PEDIATRIC STUDY.**

4 Francesco Saglio, MD¹ Silvia Cena, PhD² Massimo Berger, MD PhD¹ Paola Quarello,
5 MD PhD¹ Viola Boccasavia, PhD² Federica Ferrando, MD² Laura Pittana, MD¹
6 Benedetto Bruno, MD PhD² and Franca Fagioli MD¹

7 1. Pediatric Onco-Hematology, Stem Cell Transplantation and Cellular Therapy Division,
8 A.O.U. Citta' della Salute e della Scienza di Torino, Ospedale Infantile Regina Margherita,
9 Torino, Italy
10
11 2. Division of Hematology, A.O.U. Citta' della Salute e della Scienza di Torino, Presidio
12 Molinette, University of Torino and Department of Molecular Biotechnology and Health
13 Sciences, University of Torino, Italy.
14

15 **Corresponding author:**

16 Francesco Saglio, MD Pediatric Onco-Hematology, Stem Cell Transplantation and
17 Cellular Therapy Division, A.O.U. Citta' della Salute e della Scienza di Torino, Ospedale
18 Infantile Regina Margherita, Torino, Italy

19 Piazza Polonia 94 - 10126 Torino - Italy

20 Tel + 39 0113135449

21 Fax + 39 0113135375

22 E-mail francesco.saglio@hotmail.it

23

24

25 **Short Title:**

26 sjTREC in pediatric allogeneic hematopoietic stem cell transplantation

27 **Financial Disclosure Statement**

28 The authors have no commercial, proprietary or financial interests in the products or
29 companies described in this article.

30 **Abstract**

31 Robust T cell function recovery has been shown to be crucial in determining allogeneic
32 hematopoietic stem cell transplantation outcome and there is growing evidence that the
33 thymus plays a central role in regulating this process. We performed a long-term analysis
34 of the role of thymic activity recovery in a population of pediatric patients undergoing
35 allogeneic hematopoietic stem cell transplantation by signal joint T-cell receptor excision
36 circle quantification. In this study, characterized by a long-term follow-up (median: 72
37 months), we found that patients with higher levels of signal joint T-cell receptor excision
38 circles before transplantation had a statistically significant reduced risk of death compared
39 with patients with lower values (Relative Risk: 0.31 95% CI: 0.30-0.32 p=0.02) and we
40 showed that this different outcome was mainly related to a reduction of relapse incidence
41 (14% versus 43% p = 0.02). Unlike from previous reports we observed no correlation
42 between signal joint T-cell receptor excision circle levels and lymphocyte recovery.
43 Moreover, we confirmed that only GvHD influenced thymic activity after transplantation.
44 In conclusion, our results suggest that there is an association between pre-transplantation
45 thymic activity and the long-term outcome of pediatric patients undergoing hematopoietic
46 stem cell transplantation, mainly through a reduction of relapse opportunities

47

48 **Introduction**

49 Allogeneic Hematopoietic Stem Cells Transplantation (alloHSCT) is one of the best
50 therapeutic options available for pediatric patients affected by various malignant diseases
51 and other non-malignant disorders involving the hematopoietic system (1). T lymphocyte
52 function recovery is a crucial event in determining the prognosis of patients undergoing
53 alloHSCT as its prolonged impairment may be related to the occurrence of infectious

54 complications and, in the malignant setting, also to the recurrence of primary disease (2,
55 3). T cell recovery after alloHSCT typically evolves throughout two distinct phases called
56 thymus-independent, or early phase, and thymus-dependent, or late phase. The thymus-
57 independent phase consists in the peripheral expansion of mature T cells transferred to the
58 patient with the graft (4, 5). The thymus-dependent phase consists in the generation of new
59 naïve T cells from the donor-derived hematopoietic progenitors occurring in the recipient's
60 thymus. The thymus-dependent phase accounts for the most durable reconstitution of the
61 T-cell compartment, generates T Cell Receptor (TCR) repertoire diversity (6) and requires
62 a functionally active thymus (7). Thymic function can be evaluated through the evaluation
63 of the signal joint T-cell receptor excision circles (sjTRECs) by quantitative polymerase
64 chain reaction (PCR). sjTRECs are episomal DNA fragments resulting from the deletion
65 of the TCR δ region during TCR α locus rearrangement. As they cannot replicate and are
66 not duplicated, they are diluted out during cell division allowing a direct evaluation of
67 recent thymic output (8, 9). Some previous studies explored the relationship between
68 sjTREC levels and the kinetics of the phenotypic and functional changes in peripheral T
69 cells after alloHSCT, showing a direct correlation between sjTREC levels and the
70 percentage of naïve T cells resulting from the thymus-dependent recovery pathway in both
71 adults (10, 11) and pediatric (10-12) patients. sjTREC levels have also been associated with
72 major parameters affecting the transplantation outcome such as the incidence of acute and
73 chronic GvHD (13, 14), opportunistic infections (7, 13) and relapse (15, 16) but all these
74 studies focused on a single parameter, in one single setting at a single time point (17) and
75 in mixed (pediatric and adult) populations. In our study we conducted a long-term
76 comprehensive analysis of the impact of sjTRECs on main transplantation outcome
77 variables in a homogenous pediatric population undergoing alloHSCT.

78

79 **Materials and methods**

80 **Patients**

81 The study population included 57 patients (38 males and 19 females) aged from 0 to 22
82 years (median age: 9 years) who underwent alloHSCT between April 2006 and October
83 2008 at our Center. In order to exclude possible bias related to a too short observation
84 period, analyses were performed when the majority of patients reached a median follow up
85 of over 5 years. The Institutional Committee on Medical Ethics approved this study and
86 patients or their legal representatives provided informed consent.

87 Patients' characteristics, conditioning regimens, hematopoietic stem cell sources, donor
88 characteristics and GvHD prophylaxes are summarized in Table 1.

89 Donor selection and HLA typing were performed according to the Italian Bone Marrow
90 Donor Registry (IBMDR) Standard of Practice. In the analyses, total nucleated cells (TNC)
91 and CD34⁺ cells values were expressed in percentiles and in quartiles according to their
92 non-Gaussian distribution. Pre-transplantation co-morbidities were scored according to a
93 previously reported classification for pediatric patients (18). The patients underwent
94 clinical and hematological post-transplantation assessments according to our Center's
95 policy. Complete blood counts were performed daily until hematological recovery, twice a
96 week until day + 100 and according to the patients' clinical conditions thereafter.

97 aGvHD and cGvHD were diagnosed and classified according to previously reported
98 criteria (19, 20). To monitor patients for viral complications, cytomegalovirus (CMV),
99 Epstein-Barr virus (EBV) and adenovirus PCR were performed weekly on peripheral
100 blood.

101

102 **sjTREC Frequency Evaluation**

103 The day before starting the conditioning regimen, on days 90 ± 7 , 180 ± 7 and 365 ± 7 patients
104 were evaluated for sjTREC frequency according to previously reported method (21, 22) on
105 peripheral blood mononuclear cells (PBMC) by real time quantitative PCR (TaqMan
106 Technology). The primer TREC sequences and probes used were: forward: 5'-
107 TGGTTTTTGTGCCAC-3'; reverse: 5'- GTGCCAGCTGCAGGGTTT-3'; probe:
108 5'(FAM) CATAGGCACCTGCACCCCGTGC (TAMRA) P-3'. PCR conditions were: 2
109 minutes at 50°C, 10 minutes at 95°C followed by 45 cycles of amplification (95°C for 15
110 seconds, 60°C for 1 minute). In order to obtain absolute sjTREC quantification we prepared
111 a standard curve by using five different concentrations of a PCR2-1TA plasmid encoding
112 the sjTREC sequence. PCR was performed using the ABI PRISM 7900HT Sequence
113 Detection System (Applied Biosystem, Foster City, CA) and data obtained were analyzed
114 using SDS.2 software (Applied Biosystems, Foster City, CA). sjTREC values are
115 expressed as copy number/100 ng DNA from PBMC. As the non-Gaussian distribution of
116 sjTREC values and almost all patients enrolled in this study had median sjTREC values
117 under the median value of age-matched controls at all the time points, all analyses were
118 performed considering sjTREC percentiles and quartiles of the study population.

119

120

121

122 **Definitions and Outcome Endpoints**

123 The primary endpoint of this study is the assessment of the impact of sjTREC levels on the
124 overall survival rates in a population of pediatric patients undergoing HSCT.

125 The secondary endpoints of the study are the assessment of sjTREC levels on both
126 Transplant Related Mortality and Relapse Incidence and the identification of transplant-
127 related factors able to influence sjTREC levels. Overall survival (OS) is defined as the
128 probability of survival irrespective of the disease state at any point in time. If, at the end of
129 the study time, the patient is still alive data are censored at the last follow-up date.
130 Transplant Related Mortality (TRM) is defined as the probability of dying without a
131 previous relapse occurrence. If the patient either experienced relapse or is still alive at the
132 end of the study time, data are censored at the relapse date or at last follow-up date
133 respectively. For malignant diseases, relapse incidence (RI) is defined as the probability of
134 having had a relapse. If the patient either died without experiencing relapse or is still alive
135 at the end of the study time, data are censored at the date of death or at the last follow-up
136 date respectively. For malignancies, patients not in a first complete remission at the time
137 of transplant and patients who had previously failed at least one first-line treatment were
138 considered as being in an advanced disease phase, while all other patients were considered
139 as being in an early disease phase.

140

141 **Chimerism and immune recovery evaluation**

142 Donor chimerism was determined at $+30\pm 7$ and $+60\pm 7$ days after alloHSCT on whole bone
143 marrow mononuclear cells and at $+180\pm 7$ and $+365\pm 7$ days on PBMC by quantitative PCR
144 of informative short tandem repeats (STR) in the recipient and donor, according to a
145 previously described method (23). Absolute Lymphocyte numbers were obtained from
146 complete blood count analyses and compared to normal values according to the patient's
147 age (24). Lymphocyte recovery was defined as the first of three consecutive days with an
148 absolute lymphocyte count over the 5th percentile of normal values for the patient's age. In

149 a subset of patients, we also investigated specific lymphocyte sub-population recovery at
150 +180 days and +365 days by flow-cytometry. Helper T cell (CD3⁺CD4⁺), cytotoxic T cell
151 (CD3⁺CD8⁺), NK cell (CD16⁺CD56⁺) and B cell (CD19⁺CD20⁺) recovery was defined as
152 the presence of an absolute number of cells over the 5th percentile of normal values
153 according to the patient's age (24).

154

155 **Statistical analysis**

156 OS was calculated according to the Kaplan-Meier method and the significance between the
157 observed differences were established by the log-rank test (25).

158 The multivariate analysis on OS was performed using Cox's method.

159 TRM and Relapse rate were calculated as a cumulative incidence (CI) to adjust the analysis
160 for competing risks: relapse and transplant-related death were considered competing risks,
161 respectively. The differences in terms of CI were compared using Grey's test. To assess
162 the influence of different transplant-related variables on sjTREC levels, a two-tailed Fisher
163 Test was performed. A p-value less than 0.05 was considered statically significant. To
164 perform multivariate analyses we selected variables reaching p-values less than 0.1 in the
165 univariate analyses. All the statistical analyses were performed using SPSS (IBM Corp.
166 2012, Armonk, NY, USA), NCSS (Hintze, 2001; NCSS PASS, Number Crunched
167 Statistical System, Kaysville, UT, USA) and R 2.5.0 software packages.

168

169 **Results**

170 **sjTREC Frequency**

171 Median sjTREC values were 16 (0-1684), 1 (0-160), 14 (0-553) and 201 (0-1006) sjTREC
172 copies/100 ng DNA before HSCT and at day +90, day +180 and day +365, respectively.

173 In order to identify transplant-related factors associated to the frequency of sjTREC, we
174 evaluated the impact of different variables on median sjTREC values before HSCT and
175 then at different time points (Table 2).

176

177 **Overall Survival**

178 As at March 2014, the median follow-up time of patients who are still alive is 72 months
179 (42-90). The overall Survival (OS) rate at 7 years of the entire study population is 70%
180 (95%CI: 58-82). We found a statistically significant relationship between sjTREC
181 frequency before transplantation and 7 years OS. Patients with sjTRECs below the 50th
182 percentile of the study population values before HSCT had an OS of 56% (95%CI: 38-73),
183 while patients with sjTRECs above the 50th percentile had an OS of 85% (95%CI: 71-98)
184 (p=0.02) (Table 3 and Figure 1). Moreover, before transplantation, it was possible to
185 perform a more extended analysis considering the sjTREC frequency sub-grouped into
186 quartiles: patients with sjTREC values in the 1st, 2nd, 3rd and 4th quartiles had OS rates of
187 40% (95%CI: 14-65), 71% (95% CI: 47-94), 87% (95%CI: 69-100) and 83% (95%CI: 63-
188 100) respectively, and these differences were statistically significant (p= 0.009).
189 Considering OS at 2 years, we found that there is a difference according to pre HSCT
190 sjTREC levels: patients with sjTREC levels under the median value of the study population
191 had an OS of 73% (95% CI: 57-89), while patients with sjTREC levels over the median
192 value of the study population had an OS of 89 % (95% CI: 57-89), although at this time
193 point this difference is not statistically significant (p= 0.13). Restricting 2-years OS
194 analysis according to pre HSCT sjTREC levels only to the cohort of patients affected by
195 malignant diseases, we also highlighted a difference (p= 0,14) but not statistically
196 significant. Female patients showed better OS compared to male patients [89% (CI95%:

197 75-100) versus 60% (CI95%: 44-76) p=0.035] and patients in an early disease phase had
198 better OS compared to patients in advanced disease phases [100% versus 60% (95%CI: 44-
199 76) p= 0.04]. All the other variables investigated in the univariate analysis (Table 3)
200 showed no correlation with OS. In particular we did not observe a correlation between OS
201 or sjTREC levels at +90, +180 and +365 days after HSCT (Table 3). To perform
202 multivariate analysis we selected from among variables listed in Table 3, those reaching a
203 p value less than 0.1 in the univariate analysis (sex, co-morbidities, disease phase at HSCT
204 and pre HSCT sjTREC levels). In the multivariate analysis, sjTREC levels before
205 transplantation and pre HSCT co-morbidities were the only variables we found to be
206 associated with OS: the patients with higher sjTRECs values showed a statistically
207 significant reduced risk of death compared with patients with lower sjTRECs values
208 (Relative Risk: 0.49 95%CI: 0.48-0.5 p=0.03) and patients in low risk group according to
209 Smith et al (18) showed a statistically significant reduced risk of death compared with
210 patients in the intermediate risk group (Relative Risk: 2.5 95%CI: 2.49-2.5 p=0.03). In
211 multivariate analysis sex and the disease phase showed no statistically significant
212 relationship with OS.

213

214 **Transplant Related Mortality**

215 The overall Transplant Related Mortality (TRM) was 5% (95%CI: 2-16). In the univariate
216 analysis sjTREC levels before transplantation and sjTREC levels at +90 days did not show
217 any correlation with the TRM. At +180 days from the transplant the patients with sjTRECs
218 values under the 50th percentile had TRM of 11% (95%CI: 4-32) versus TRM of 0 of
219 patients with sjTREC values over the 50th percentile. Likewise, at +365 days patients with
220 sjTREC values under the 50th percentile had TRM of 10% (95%CI: 1-37) versus TRM of

221 0 of patients with sjTREC values over the 50th percentile. These differences in terms of
222 TRM were not statistically significant ($p= 0.1$ and $p= 0.17$ respectively) (Table 4).

223

224 **Relapse Incidence**

225 For malignant disease, the overall Relapse Incidence (RI) was 30% (95% CI: 20-46).

226 sjTREC levels before transplantation were related to the relapse. Patients with sjTREC
227 levels below the 50th percentile of the study population relapsed in 43% of cases (95% CI:
228 28-66), while 14% patients with sjTREC levels above the 50th percentile experienced a
229 relapse (95% CI: 5-41) and this difference was statistically significant ($p= 0.02$) (Table 4
230 and Figure 2). Considering sjTREC levels before the transplant sub-grouped in quartiles,
231 patients with sjTREC levels in the 1st, 2nd, 3rd and 4th quartiles had a relapse in 64% (95%
232 CI: 43-95), 21% (95% CI: 8-58), 14% (95% CI: 4-51) and 14% (95% CI: 2-88) of cases,
233 respectively, and this difference was statistically significant ($p=0.01$). sjTREC levels at
234 +90, +180 and +365 days were not related to the recurrence. Among other variables
235 investigated by univariate analysis the patient's gender showed a relationship with RI: male
236 patients relapsed in 40% (95% CI: 27-60) while female patients relapsed in 7% (95% CI:
237 1-47) $p= 0.03$ (Table 5). To perform multivariate analysis, we selected, from among the
238 variables listed in Table 5, those reaching a p value less than 0.1 in the univariate analysis
239 (sex and pre-HSCT sjTREC levels) and disease phase at HSCT. In the multivariate analysis
240 sjTREC levels before transplantation were the only variables we found to be statistically
241 associated (RR 0 0 $p < 0.0001$) with RI.

242

243 **Chimerism and Immune Recovery**

244 All the patients showed sustained engraftment and we did not observe any cases of either

245 early- or late-graft loss. Patients enrolled in the study reached the 5th percentile of normal
246 lymphocyte values for the patient's age in a median of 70 days (range: 21-420) with no
247 differences related to pre-HSCT sjTREC levels: 73 days (range: 25-420) for patients with
248 sjTRECs over the 50th percentile before HSCT versus 65 days (range: 21-385) for patients
249 with sjTRECs under the 50th percentile before HSCT. Considering the lymphocyte
250 subpopulations, the proportion of patients who reached the 5th percentile of normal values
251 for their ages of CD3⁺CD4⁺, CD3⁺CD8⁺, CD16⁺CD56⁺ and CD19⁺CD20⁺ cells was 17%,
252 65%, 82% and 60% at day +180 and 70%, 85%, 88% and 77% at day +365, respectively,
253 with no differences related to pre-HSCT sjTREC levels.

254

255 **Discussion**

256 T cell function recovery has been shown to be one of the most important factors in
257 determining the prognosis of patients undergoing alloHSCT and the role of the thymus in
258 this process is well established. Previous studies focused on Severe Combined
259 Immunodeficiency Disease Screening Programs in newborns (26) and on the
260 management of patients affected by HIV and undergoing Highly Active Antiretroviral
261 Therapy (HAART) (27) indicate that sjTREC quantification is an easy, sensible and
262 reliable technique to evaluate immunological function and also to drive therapeutic
263 interventions in these settings. Although the experience of alloHSCT is more limited,
264 there is growing evidence that sjTREC quantification by PCR is one of the easiest and
265 most reliable methods to evaluate thymic activity after alloHSCT as well. This is because,
266 compared to other techniques (i.e. flow cytometry), this method offers the advantage of
267 not being influenced by any phenomena which typically occur after transplantation, such

268 as the opportunity of T memory cells to revert into a T naïve phenotype in case of
269 recurrent herpes virus infection (28), the possibility of T naïve cells to maintain their
270 phenotype while acquiring T memory cells' function (29) and the maintenance of CD31
271 expression during CD4⁺ cells cytokine-driven proliferation (30). Even though other
272 studies have already shown that there is a correlation between sjTREC levels and the
273 various phases of immune recovery after alloHSCT (10-12) and that patients with a more
274 efficient thymic function show a better prognosis compared to others (13, 31), there are
275 still very few studies specifically concerning pediatric patients and considering that aging
276 is a major parameter impacting thymic function (9, 32), childhood may be considered an
277 ideal setting to further consolidate these data. In the present study we analyzed in a
278 population of pediatric patients undergoing alloHSCT, the role of sjTREC levels on the
279 OS and found that patients with more efficient thymic function before the transplantation
280 had better long term OS compared to others. However, sjTREC levels after
281 transplantation, according to our data, did not have any influence on OS at any of the
282 time-points considered. To our knowledge there are only two previous studies that
283 specifically investigate the impact of sjTRECs on OS. Clave *et al* demonstrated a
284 correlation between pre-transplantation sjTREC levels and OS but even though a high
285 number of cases were reported (n=102), only sibling recipients were included, the
286 patients' median age was higher and only pre-transplantation sjTREC levels (17) were
287 considered. Olkinuora *et al* in 66 pediatric patients who underwent alloHSCT reported a
288 shorter median survival time for patients with low sjTREC levels at different time points
289 (both before and after alloHSCT) compared to patients with high sjTREC levels, but their
290 study lacked a real survival analysis performed with the Kaplan-Meier method and the
291 follow-up is shorter (33). We basically confirmed a correlation between sjTREC levels

292 and OS in a more homogeneous and younger population also including unrelated
293 transplant recipients and, by extending the follow-up to a median time of 72 months, we
294 highlighted that among pediatric patients long-term survival is closely related to pre-
295 HSCT sjTREC levels. However, as a large proportion of the patients enrolled in our study
296 have acute lymphoblastic leukemia (ALL) that tends to relapse in the first months after
297 transplantation, the correlation between sjTREC levels and OS seems to be less strong in
298 the short term moreover the small number of patients affected by non malignant disorders
299 included in the study population might introduce some potential confounding factors that
300 are to be considered. In order to understand whether the mortality reduction we observed
301 was attributable to a reduction of either TRM or RI, we analyzed in the same population
302 the impact of sjTREC levels on these two outcome parameters. In line with other authors'
303 findings, we observed a strong correlation between pre-transplantation thymic functions
304 and RI (15, 16). However, unlike these authors, who investigated the role of sjTRECs in
305 only one specific setting, surprisingly, we did not observe a correlation between post-
306 transplantation thymic activity and RI. This difference might be related to the
307 heterogeneity of our study population that included bone marrow, peripheral blood stem
308 cells and cord blood recipients. One possible objection to our observations might be that
309 reduced sjTREC frequency before alloHSCT might be related to more intense treatments
310 administered because of a more aggressive disease and that OS and RI differences might
311 only be related to a more advanced disease phase. However, via multivariate analysis we
312 were able to show how sjTREC levels before transplantation are statistically associated to
313 OS and RI independently from other variables, including the presence of an advanced
314 disease phase, and, by correlation analysis, we excluded a link between the disease phase
315 at transplantation and the time between diagnosis and HSCT and sjTREC frequency

316 before alloHSCT. In multivariate analysis we found that the only other variable
317 associated with OS was the presence of co-morbidities as scored by Smith (18). Unlike
318 previous observations (7, 13, 14, 33, 34), we did not observe a relationship between
319 sjTRECs and TRM, probably because the very low incidence of these complications in
320 our study population, related to the lower frequency of co-morbidities in young
321 individuals. Finally, to clarify whether increased OS and reduced RI of patients with
322 higher pre-HSCT sjTREC levels were related to an improved immune recovery, we
323 evaluated the absolute lymphocyte count recovery and, surprisingly, we did not observe
324 any differences between the patients with values over the 50th percentile or patients with
325 values under the 50th percentile. Our data contrast with previous reports (12) but this
326 might be due to our smaller sample size and might be related to the differences in the
327 recovery of different lymphocyte subsets (T, B, NK) after HSCT. By analyzing transplant
328 related factors that influence sjTREC frequency, we confirmed previously reported
329 observations on adults (35) confirming GvHD as one of the most limiting factors in
330 determining sjTREC levels after transplantation. However, according to our analysis,
331 sjTREC reductions after HSCT was not statistically correlated with any worsening in
332 terms of OS, TRM or Relapse.

333 The main weakness of our study is that we analyzed sjTREC frequency on whole PBMCs
334 while other authors performed the same analysis more precisely on selected lymphocyte
335 populations (i.e. CD3⁺, CD3⁺ CD4⁺, CD3⁺ CD8⁺).

336 Another limit of our study is that in the series of patients we have described, the majority
337 of the patients was affected by ALL but no cases of T cell leukemia were included and
338 this may have some consequences in terms of both RI and OS.

339 In conclusion our results confirm that thymic function does play an important role in
340 determining the prognosis of pediatric patients undergoing alloHSCT, suggesting that an
341 efficient thymic function before transplantation is related to improved long-term OS,
342 mainly through a reduction of relapse opportunities. Obviously, larger and more accurate
343 studies are needed both to confirm these observations and to identify the mechanism
344 driving them, in order to find solutions aimed at improving T cell recovery after
345 alloHSCT.

346

347 **Acknowledgments**

348 This work was supported by Associazione Donatrici Italiane Sangue Cordone Ombelicale
349 (ADISCO) sezione Piemonte, Progetti di Ricerca ex-60%, Regione Piemonte: Ricerca
350 Finalizzata 2008, 2009; Comitato Regionale Piemontese Gigi Ghirotti and Associazione
351 Italiana contro le Leucemie, i Linfomi e il Mieloma, Sezione di Torino.

352 We are grateful to Dr Chiara Bonini and Dr Alessandro Aiuti from IRCCS San Raffaele,
353 Milan, Italy, for providing a PCR2-ITA plasmid encoding the sjTREC sequence and for
354 scientific advice and to Mr Andrew Martin Garvey, BA(Hons), LTCL, MA for patiently
355 reviewing our paper.

356 **References**

- 357 1. Miano M, Labopin M, Hartmann O, Angelucci E, Cornish J, Gluckman E, et al.
358 Haematopoietic stem cell transplantation trends in children over the last three decades: a
359 survey by the paediatric diseases working party of the European Group for Blood and
360 Marrow Transplantation. *Bone Marrow Transplant.* 2007 Jan;39(2):89-99.

- 361 2. Peggs KS, Mackinnon S. Immune reconstitution following haematopoietic stem
362 cell transplantation. *Br J Haematol.* 2004 Feb;124(4):407-20.
- 363 3. Fallen PR, McGreavey L, Madrigal JA, Potter M, Ethell M, Prentice HG, et al.
364 Factors affecting reconstitution of the T cell compartment in allogeneic haematopoietic
365 cell transplant recipients. *Bone Marrow Transplant.* 2003 Nov;32(10):1001-14.
- 366 4. Mackall CL, Hakim FT, Gress RE. T-cell regeneration: all repertoires are not
367 created equal. *Immunol Today.* 1997 May;18(5):245-51.
- 368 5. Mackall CL, Bare CV, Granger LA, Sharrow SO, Titus JA, Gress RE. Thymic-
369 independent T cell regeneration occurs via antigen-driven expansion of peripheral T cells
370 resulting in a repertoire that is limited in diversity and prone to skewing. *J Immunol.* 1996
371 Jun 15;156(12):4609-16.
- 372 6. Mackall CL, Granger L, Sheard MA, Cepeda R, Gress RE. T-cell regeneration
373 after bone marrow transplantation: differential CD45 isoform expression on thymic-
374 derived versus thymic-independent progeny. *Blood.* 1993 Oct 15;82(8):2585-94.
- 375 7. Wils EJ, van der Holt B, Broers AE, Posthumus-van Sluijs SJ, Gratama JW,
376 Braakman E, et al. Insufficient recovery of thymopoiesis predicts for opportunistic
377 infections in allogeneic hematopoietic stem cell transplant recipients. *Haematologica.*
378 2011 Dec;96(12):1846-54.
- 379 8. Dion ML, Sekaly RP, Cheynier R. Estimating thymic function through
380 quantification of T-cell receptor excision circles. *Methods Mol Biol.* 2007;380:197-213.
- 381 9. Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, et al.
382 Changes in thymic function with age and during the treatment of HIV infection. *Nature.*
383 1998 Dec 17;396(6712):690-5.

- 384 10. Douek DC, Vescio RA, Betts MR, Brenchley JM, Hill BJ, Zhang L, et al.
385 Assessment of thymic output in adults after haematopoietic stem-cell transplantation and
386 prediction of T-cell reconstitution. *Lancet*. 2000 May 27;355(9218):1875-81.
- 387 11. Dumont-Girard F, Roux E, van Lier RA, Hale G, Helg C, Chapuis B, et al.
388 Reconstitution of the T-cell compartment after bone marrow transplantation: restoration
389 of the repertoire by thymic emigrants. *Blood*. 1998 Dec 1;92(11):4464-71.
- 390 12. Chen X, Barfield R, Benaim E, Leung W, Knowles J, Lawrence D, et al.
391 Prediction of T-cell reconstitution by assessment of T-cell receptor excision circle before
392 allogeneic hematopoietic stem cell transplantation in pediatric patients. *Blood*. 2005 Jan
393 15;105(2):886-93.
- 394 13. Lewin SR, Heller G, Zhang L, Rodrigues E, Skulsky E, van den Brink MR, et al.
395 Direct evidence for new T-cell generation by patients after either T-cell-depleted or
396 unmodified allogeneic hematopoietic stem cell transplantations. *Blood*. 2002 Sep
397 15;100(6):2235-42.
- 398 14. Talvensaari K GF, Busson M. . Pretransplant thymic function could have a
399 predictive value for the incidence of graft vs host disease and general outcome after
400 allogeneic bone marrow transplantation [abstract]. *Blood*. 2001;98:
401 396a. *Blood* 2001;98: 396a.
- 402 15. Clave E, Lisini D, Douay C, Giorgiani G, Busson M, Zecca M, et al. A low
403 thymic function is associated with leukemia relapse in children given T-cell-depleted
404 HLA-haploidentical stem cell transplantation. *Leukemia*. 2012 Aug;26(8):1886-8.

- 405 16. Clave E, Lisini D, Douay C, Giorgiani G, Busson M, Zecca M, et al. Thymic
406 function recovery after unrelated donor cord blood or T-cell depleted HLA-haploidentical
407 stem cell transplantation correlates with leukemia relapse. *Front Immunol.* 2013;4:54.
- 408 17. Clave E, Rocha V, Talvensaari K, Busson M, Douay C, Appert ML, et al.
409 Prognostic value of pretransplantation host thymic function in HLA-identical sibling
410 hematopoietic stem cell transplantation. *Blood.* 2005 Mar 15;105(6):2608-13.
- 411 18. Smith AR, Majhail NS, MacMillan ML, DeFor TE, Jodele S, Lehmann LE, et al.
412 Hematopoietic cell transplantation comorbidity index predicts transplantation outcomes
413 in pediatric patients. *Blood.* 2011 Mar 3;117(9):2728-34.
- 414 19. Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J, et al.
415 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant.* 1995
416 Jun;15(6):825-8.
- 417 20. Shulman HM, Sullivan KM, Weiden PL, McDonald GB, Striker GE, Sale GE, et
418 al. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20
419 Seattle patients. *Am J Med.* 1980 Aug;69(2):204-17.
- 420 21. Sodora DL, Douek DC, Silvestri G, Montgomery L, Rosenzweig M, Igarashi T, et
421 al. Quantification of thymic function by measuring T cell receptor excision circles within
422 peripheral blood and lymphoid tissues in monkeys. *European journal of immunology.*
423 2000 Apr;30(4):1145-53.
- 424 22. Hazenberg MD, Verschuren MC, Hamann D, Miedema F, van Dongen JJ. T cell
425 receptor excision circles as markers for recent thymic emigrants: basic aspects, technical
426 approach, and guidelines for interpretation. *Journal of molecular medicine.* 2001
427 Nov;79(11):631-40.

- 428 23. Thiede C, Florek M, Bornhauser M, Ritter M, Mohr B, Brendel C, et al. Rapid
429 quantification of mixed chimerism using multiplex amplification of short tandem repeat
430 markers and fluorescence detection. *Bone Marrow Transplant.* 1999 May;23(10):1055-
431 60.
- 432 24. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC,
433 Groeneveld K, et al. Immunophenotyping of blood lymphocytes in childhood. Reference
434 values for lymphocyte subpopulations. *J Pediatr.* 1997 Mar;130(3):388-93.
- 435 25. Mantel N. Evaluation of survival data and two new rank order statistics arising in
436 its consideration. *Cancer chemotherapy reports Part 1.* 1966 Mar;50(3):163-70.
- 437 26. Baker MW, Grossman WJ, Laessig RH, Hoffman GL, Brokopp CD, Kurtycz DF,
438 et al. Development of a routine newborn screening protocol for severe combined
439 immunodeficiency. *The Journal of allergy and clinical immunology.* 2009
440 Sep;124(3):522-7.
- 441 27. Saitoh A, Singh KK, Sandall S, Powell CA, Fenton T, Fletcher CV, et al.
442 Association of CD4+ T-lymphocyte counts and new thymic emigrants in HIV-infected
443 children during successful highly active antiretroviral therapy. *The Journal of allergy and*
444 *clinical immunology.* 2006 Apr;117(4):909-15.
- 445 28. Bouneaud C, Garcia Z, Kourilsky P, Pannetier C. Lineage relationships,
446 homeostasis, and recall capacities of central- and effector-memory CD8 T cells in vivo. *J*
447 *Exp Med.* 2005 Feb 21;201(4):579-90.
- 448 29. Bains I, Antia R, Callard R, Yates AJ. Quantifying the development of the
449 peripheral naive CD4+ T-cell pool in humans. *Blood.* 2009 May 28;113(22):5480-7.

- 450 30. Toubert A, Glauzy S, Douay C, Clave E. Thymus and immune reconstitution after
451 allogeneic hematopoietic stem cell transplantation in humans: never say never again.
452 *Tissue Antigens*. 2012 Feb;79(2):83-9.
- 453 31. Hochberg EP, Chillemi AC, Wu CJ, Neuberg D, Canning C, Hartman K, et al.
454 Quantitation of T-cell neogenesis in vivo after allogeneic bone marrow transplantation in
455 adults. *Blood*. 2001 Aug 15;98(4):1116-21.
- 456 32. Lynch HE, Goldberg GL, Chidgey A, Van den Brink MR, Boyd R, Sempowski
457 GD. Thymic involution and immune reconstitution. *Trends Immunol*. 2009
458 Jul;30(7):366-73.
- 459 33. Olkinuora H, Talvensaaari K, Kaartinen T, Siitonen S, Saarinen-Pihkala U,
460 Partanen J, et al. T cell regeneration in pediatric allogeneic stem cell transplantation.
461 *Bone Marrow Transplant*. 2007 Feb;39(3):149-56.
- 462 34. Brown JA, Stevenson K, Kim HT, Cutler C, Ballen K, McDonough S, et al.
463 Clearance of CMV viremia and survival after double umbilical cord blood transplantation
464 in adults depends on reconstitution of thymopoiesis. *Blood*. 2010 May 20;115(20):4111-
465 9.
- 466 35. Weinberg K, Blazar BR, Wagner JE, Agura E, Hill BJ, Smogorzewska M, et al.
467 Factors affecting thymic function after allogeneic hematopoietic stem cell transplantation.
468 *Blood*. 2001 Mar 1;97(5):1458-66.

469

470

471 **Tables**472 **Table 1. Patients' and Hematopoietic Stem Cell Transplantation details**

473

		n	%
Sex	Male	38	67 %
	Female	19	33 %
Disease	ALL	23	40 %
	AML	8	14 %
	Inborn errors	6	10 %
	Solid Tumors	6	10 %
	Lymphoma	5	9 %
	MDS & JMML	4	7 %
	HLH	2	3,5 %
	SAA	2	3,5 %
	CML	1	1 %
	Phase*	Early	8
Advanced		39	83 %
Co-morbidity score (18)	0	44	79 %
	1-2	13	23 %
	3+	0	
Conditioning Regimen	TBI based	31	54 %
	Bu based	13	23 %
	Others	13	23 %
HSC source	BM	46	81 %
	CB	8	14 %
	PBSC	3	5 %
Donor	Sibling	21	37 %
	MUD	17	30 %
	MMUD	11	19 %
	CB	8	14 %
GvHD prophylaxis	CyA-MTX-ATG	27	48 %
	CyA	12	21 %
	CyA-MTX	8	14 %
	CyA-ATG-MMF	4	7 %
	CyA-ATG-PDN	3	5 %
	Others	3	5 %

474

475 *for malignant diseases only

476 ALL: Acute Lymphoblastic Leukemia, AML: Acute Myelogenous Leukemia, MDS: myelodysplasia, JMML: Juvenile Myelo
477 Monocytic Leukemia, HLH: Hemophagocytic Lymphohistiocytosis SAA: Severe Aplastic Anemia, CML: Chronic Myelogenous
478 Leukemia, TBI: Total Body Irradiation, Bu: busulfan, BM: bone marrow, CB: cord blood, PBSC: peripheral blood stem cells, MUD:
479 matched unrelated donor, MMUD: mismatched unrelated donor, CyA: cyclosporine, MTX: methotrexate, ATG: antithymocyte
480 globulins, MMF: mycophenolate mofetil, PDN: prednisone.

481

482

483

484 **Table 2. sjTREC frequency**

485
486
487
488
489

HSCT hematopoietic stem cell transplantation, ATG: antithymocyte globulins *for malignant diseases only

		No. of patients with sjTREC level < of median value of the study population	No. of patients with sjTREC level > of the median value of the study population	p
Pre-HSCT (pts evaluable 57)	Age			
	0-5 years (n=15)	6 (40%)	9 (60%)	
	6-8 years (n=11)	3 (27%)	8 (73%)	
	9-14 years (n= 16)	8 (50%)	8 (50%)	
	>14 years (n= 15)	8 (53%)	7 (47%)	
	Disease			0.03
	Malignant (n= 47)	28 (60%)	19 (40%)	
	Non-malignant (n= 10)	2 (20%)	8 (80%)	
	Comorbidities(18)			0.21
	Low risk (n=44)	21 (48%)	23 (52%)	
	Intermediate risk (n= 13)	9 (70%)	4 (30%)	
	Disease Phase*			0.005
	Early (n= 8)	1 (12%)	7 (88%)	
	Advanced (n=39)	27 (69%)	12 (31%)	
Time from diagnosis to HSCT			0.57	
< 6 months (n=25)	16 (64%)	9 (36%)		
> 6 months (n=22)	12 (54%)	10 (46%)		
Day +90 (pts evaluable 57)	ATG			0.02
	Yes (n= 37)	25 (68%)	12 (32%)	
	No (n= 20)	5 (25%)	15 (75%)	
	Viral Infection			0.01
Yes (n=30)	21 (70%)	9 (30%)		
No (n= 27)	9 (33%)	18 (67%)		
Day + 180 (pts evaluable 57)	grade II-IV acute GvHD			0.03
	Yes (n=21)	15 (71%)	6 (29%)	
	No (n= 36)	16 (44%)	20 (56%)	
Day + 365 (pts evaluable 43)	Age			0.03
	0-5 years (n=9)	3 (33%)	6 (64%)	
	6-8 years (n=10)	3 (30%)	7 (70%)	
	9-14 years (n=14)	7 (50%)	7 (50%)	
	>14 years (n=10)	9 (90%)	1 (10%)	
	cGvHD			0.02
	Yes (n= 6)	6 (100%)	0	
	No (n= 37)	16 (43%)	21 (57%)	
Viral Infection			0.03	
Yes (n=24)	16 (67%)	8 (33%)		
No (n= 19)	6 (32%)	13 (68%)		

490
491

Table 3. Overall Survival

Variable	n	Events	7 years OS	95% CI	Log Rank Test
<i>sjTREC</i>s pre HSCT					
<50 th percentile	30	13	56 %	(38-73)	p= 0.02
>50 th percentile	27	4	85 %	(71-98)	
<i>sjTREC</i>s +90 days					
<50 th percentile	30	9	70 %	(54-86)	p= 0.97
>50 th percentile	27	8	70 %	(52-88)	
<i>sjTREC</i>s +180 days					
<50 th percentile	29	7	60 %	(42-78)	p= 0.1
>50 th percentile	25	8	80 %	(66-94)	
<i>sjTREC</i>s +365 days					
<50 th percentile	20	4	77 %	(59-95)	p= 0.6
>50 th percentile	18	6	83 %	(65-100)	
Sex					
Male	38	15	60 %	(44-76)	p= 0.035
Female	19	2	89 %	(75-100)	
Age					
0-5 years	15	5	63 %	(36-90)	p= 0.28
6-8 years	11	4	64 %	(36-91)	
9-14 years	16	2	87 %	(71-100)	
> 15 years	15	6	60 %	(34-85)	
Disease					
Malignant	47	16	65 %	(53-81)	p= 0.14
Non-malignant	10	1	90 %	(63-100)	
Co-morbidity score					
Low risk group	44	7	84 %	(83-84)	p< 0.0001
Intermediate risk group	13	10	23 %	(0-46)	
Disease phase*					
Early	8	0	100 %		p= 0.04
Advanced	39	16	60 %	(44-76)	
Time between diagnosis and HSCT*					
< 6 months	25	7	73 %	(56-90)	p = 0.55
> 6 months	22	9	60 %	(40-80)	
TBI					
Yes	31	8	73 %	(57-89)	p= 0.51
No	26	9	65 %	(47-83)	
HSC source					
BM	46	16	65 %	(51-79)	p= 0.27
PBSC	3	1	87 %	(63-100)	
CB	8	0	100 %		
TNC					
<50 th percentile	29	9	69 %	(51-87)	p= 0.65
>50 th percentile	28	8	71 %	(53-89)	
CD34⁺ cells					
<50 th percentile	30	9	70 %	(54-86)	p= 0.78
>50 th percentile	27	8	69 %	(51-87)	

492
493
494
495

All the variables potentially able to influence OS were evaluated: sjTREC levels before alloHSCT patient's sex, co-morbidities and disease phase showed a statistically significant ($p < 0.05$) correlation with OS. sjTREC_s: signal joint T cell receptor excision circles, TBI: Total Body Irradiation, HSC: hematopoietic stem cells, BM: bone marrow, PBSC: peripheral blood stem cells, CB: cord blood, TNC: total nucleated cells

496 **Table 4. Transplant-related mortality (TRM) univariate analysis**

Variable	TRM	95% CI	Grey test
<i>sjTRECs pre-HSCT</i>			p = 0.46
<50 th percentile	3 %	(0-23)	
>50 th percentile	7 %	(2-28)	
<i>sjTRECs +90 days</i>			p = 0.60
<50 th percentile	7 %	(2-25)	
>50 th percentile	4 %	(0-26)	
<i>sjTRECs +180 days</i>			p= 0.10
<50 th percentile	11 %	(4-32)	
>50 th percentile	0		
<i>sjTRECs +365 days</i>			p= 0.17
<50 th percentile	10 %	(1-37)	
>50 th percentile	0		

497

498

499 Single joint T cells receptor excision circles (sjTRECs) level showed no statistically significant correlation (p< 0.05) with TRM at any
 500 of the time points considered

501

502

503

504

505 **Table 5. Relapse Incidence for malignant diseases: univariate analysis**

506

Variable	n	Events	Relapse Incidence	95% CI	Grey test
<i>sjTRECs pre-HSCT</i>					
<50 th percentile	28	12	43%	(28-66)	p = 0.02
>50 th percentile	19	3	14%	(5-41)	
<i>sjTRECs +90 days</i>					
<50 th percentile	26	7	26%	(14-49)	p = 0.60
>50 th percentile	21	8	33%	(18-61)	
<i>sjTRECs +180 days</i>					
<50 th percentile	27	10	37%	(15-52)	p = 0.34
>50 th percentile	20	5	25%	(5-46)	
<i>sjTRECs +365 days</i>					
<50 th percentile			11%	(3-41)	p = 0.36
>50 th percentile			23%	(8-62)	
<i>Sex</i>					
Male	34	14	41%	(27-61)	p= 0.03
Female	13	1	8 %	(1-50)	
<i>Age</i>					
0-5 years	12	4	33 %	(14-69)	p= 0.58
6-8 years	8	4	50%	(21-92)	
9-14 years	15	3	20 %	(7-55)	
> 15 years	12	4	33 %	(15-74)	
<i>Disease phase</i>					
Early	8	1	12%	(2-78)	p= 0.20
Advanced	39	14	35%	(22-52)	
<i>Time between diagnosis and HSCT</i>					
< 6 months	25	7	28%	(15-52)	p= 0.67
> 6 months	22	8	36%	(21-63)	
<i>HSC source</i>					
BM	39	14	36%	(24-55)	p= 0.29
CB	7	1	14%	(2-87)	
PBSC	1	0	0		
<i>Donor</i>					
Related	17	7	41%	(23-73)	p= 0.24
Unrelated	30	8	27%	(15-48)	
<i>aGvHD</i>					
Yes	20	5	25%	(12-53)	p= 0.41
No	27	15	37%	(23-60)	
<i>cGvHD</i>					
Yes	8	2	25%	(7-83)	p= 0.57
No	39	13	32%	(20-50)	

507

508
509
510
511

Univariate analysis of variables potentially able to influence Relapse Incidence : single joint T cell receptor excision circles (sjTREC) before the transplantation and patients' sex were statistically related to RI incidence (p < 0.05). HSC: hematopoietic stem cell, BM: bone marrow, PBSC: peripheral blood stem cells, CB: cord blood, aGvHD: acute graft versus host disease, cGvHD: chronic graft versus host disease

512 **Figure legends**

513

514 **Figure 1. Overall Survival according to sjTREC levels**

515 Patients with sjTRECs over the 50th percentile before HSCT (continuous line) showed a
516 statistically significant increased survival rate compared to patients with sjTRECs under
517 the 50th percentile (dotted line) at same time point.

518

519 **Figure 2. Relapse rate according to sjTREC levels**

520 Patients with sjTRECs over the 50th percentile before HSCT (continuous line) showed a
521 statistically significant reduced relapse rate compared to patients with sjTRECs under the
522 50th percentile (dotted line) at same time point.

523

524

525

526

527

528

529

530

531

532

533

534

535 **Figures**

536

537

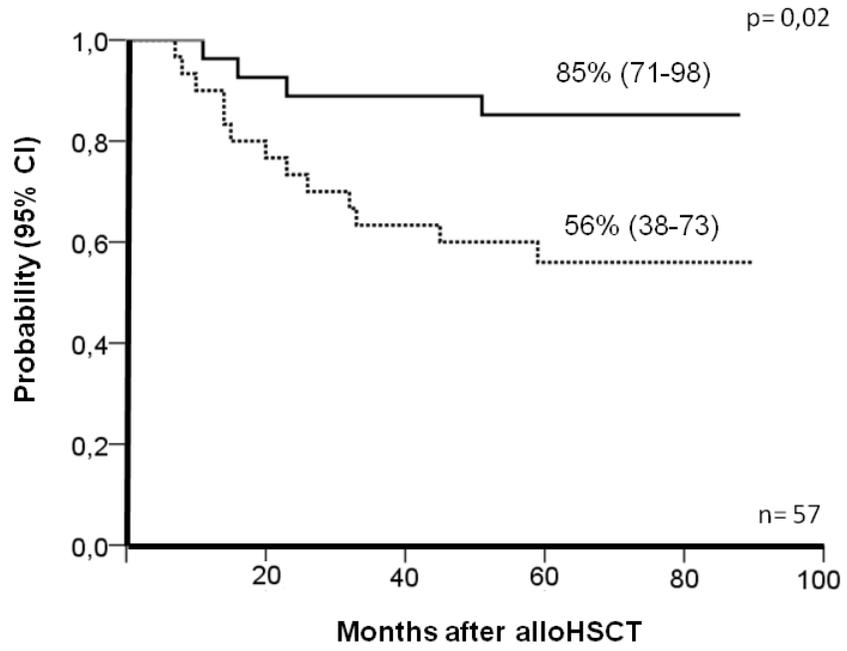


Figure 1

538

539

540

541

542

543

544

545

546

547

548

549

550

551

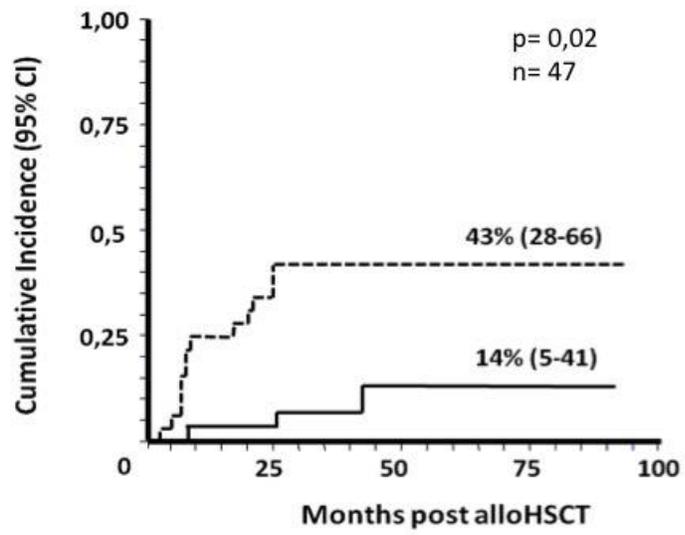


Figure 2

552

553