

# Long-term event-free survival, chimerism and fertility outcomes in 234 patients with sickle-cell anemia younger than 30 years after myeloablative conditioning and matched-sibling transplantation in France



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## ABSTRACT

Allogeneic stem cell transplantation remains the only curative treatment for sickle cell anemia (SCA), but the place of myeloablative conditioning in the procedure remains to be defined. The aim of the present study was to analyze long-term outcomes, including chimerism, SCA-related events and biological data (hemoglobin, reticulocytes, HbS%), and fertility in a French series of 234 SCA patients under 30 years of age who, from 1988 to 2012, received a matched-sibling-donor stem cell transplantation following standardized myeloablative conditioning [busulfan, cyclophosphamide and rabbit anti-thymocyte globulin (ATG)]. Since the first report of the series (1988-2004), 151 new consecutive patients with SCA have been similarly transplanted. Considering death, non-engraftment or rejection (donor cells <5%) as events, the 5-year event-free survival was 97.9% (95% confidence interval: 95.5-100%), confirming, since the year 2000, an at least 95% chance of cure. In the overall cohort (n=234, median follow up 7.9 years), event-free survival was not associated with age, but chronic-graft-versus-host disease (cGvHD) was independently associated with recipient's age >15 years (hazard ratio=4.37; P=0.002) and lower (5-15 vs. 20 mg/kg) ATG dose (hazard ratio=4.55; P=0.001). At one year, 44% of patients had mixed chimerism (5-95% donor cells), but those prepared with ATG had no graft rejection. No events related to SCA occurred in patients with mixed chimerism, even those with 15-20% donor cells, but hemolytic anemia stigmata were observed with donor cells <50%. Myeloablative transplantation with matched-sibling donor currently has a higher event-free survival (98%) in patients under 30 years of age than that reported for non-myeloablative conditioning (88%). Nevertheless, the risk of cGvHD in older patients and the need to preserve fertility might be indications for a non-myeloablative conditioning.

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## Introduction

Sickle cell anemia (SCA) represents a growing global health problem. Over 300,000 children are born each year with SCA worldwide, with 85% of these births occurring in sub-Saharan Africa.<sup>1</sup> SCA is a severe recessive genetic disorder, resulting from a single nucleotide substitution in codon 6 of the beta-globin gene, producing abnormal hemoglobin (HbS) that is prone to polymer formation under deoxygenated conditions. Polymerized HbS leads to decreased red blood cell deformability and sickling within end arterioles, resulting in vaso-occlusive crisis and pain.

Despite significant progress in the management of SCA, such as the prevention of pneumococcal infection,<sup>2,3</sup> the introduction of hydroxyurea therapy (HU),<sup>4-10</sup> and early detection of cerebral vasculopathy with transcranial Doppler,<sup>11,12</sup> followed by rapid establishment of transfusion programs for patients at risk of stroke,<sup>13,14</sup> SCA remains a disease with a high risk of morbidity and early death.<sup>15-18</sup>

Allogeneic hematopoietic stem cell transplantation (SCT) is the only curative therapy for SCD<sup>19-26</sup> as it can prevent SCA-related organ damage if the erythroid compartment is adequately replaced by donor erythropoiesis. However, barriers to SCT use include the risks of rejection, transplant-related mortality (TRM), chronic graft-versus-host disease (cGvHD), infertility, and the lack of matched-sibling donors (MSD). While at least 1,000 MSD-SCT have been performed so far worldwide, the number of transplanted SCA patients remains very low,<sup>23</sup> especially in developing countries that have a large SCA population. We have previously reported studies in 87 consecutive myeloablative MSD-SCT for SCA patients performed in France between 1988 and December 2004.<sup>22</sup> We showed that the addition of antithymocyte globulin (ATG) to the conditioning regimen (CR) allowed a significant reduction in the risk of rejection despite a higher prevalence of mixed chimerism. The myeloablative CR (MAC), consisting of busulfan, cyclophosphamide and rabbit ATG was well tolerated by these young patients (aged 2.2-22 years), as only one death occurred during aplasia and limited early toxicity was noted. Moreover, the outcome significantly improved with time, as event-free survival (EFS) was 95.3% at five years for the 44 patients transplanted between January 2000 and December 2004.<sup>22</sup> These results led us to use the same MAC in SCA adults under the age of 30 years without major organ dysfunction and children with less severe cerebral vasculopathy.

Since that initial report, extremely interesting results have been obtained in adults using non-myeloablative (NMA) CR, resulting in 87% EFS with 13% rejection risk, but with preserved fertility and no GvHD with mixed chimerism.<sup>27-29</sup> It is thus timely and warranted to report the long-term outcome of chimerism, cGvHD and fertility after MAC-MSD-SCT in order to provide evidence for making an informed decision about the use of MAC or NMA CR for SCA children and young adults. For this reason, we analyzed the results of MAC-MSD-SCT performed in France between 1988 and December 2012 in 234 SCA patients, ranging in age from 2.2 to 28.9 years with a minimum 5-year follow up for surviving patients, to evaluate long-term outcomes, especially in the context of incidence of cGvHD and chimerism.

## Methods

Considering the better cure rate observed with MSD-SCT after MAC for SCA patients transplanted after 2000 (95.3%), the Société Française de Greffe de Moelle et de Thérapie Cellulaire (SFGM-TC) decided to continue the previously described protocol,<sup>22</sup> and 151 new consecutive patients were transplanted between January 2005 and December 2012 for severe SCA. Thus, a total of 234 SCA patients had received MSD-SCT following MAC between November 1988 and December 2012. Informed consent was obtained from recipients, donors and their parents or guardians before transplantation. The data were obtained in the context of consensual treatment guidelines among French transplant centers in accordance with the Declaration of Helsinki, and the French laws and regulations protecting human subjects. To complement the data from the European Group for Blood and Marrow Transplantation (EBMT) registry, clinical and biological data including chimerism, eventual carcinogenic issues and fertility were recorded in a French database, and its use for this project was approved by the Créteil Institutional Review Board.

Patients' characteristics are presented in Table 1. The overall median age at transplant was 8.4 years (range: 2.2-28.9) with 32 patients (13.7%) older than 15 years (Figure 1A). All donors were MSD and genotype, available in 208 of 234 patients, was heterozygous AS (n=127), AThal (n=2), AC (n=1), A/DPunjab (n=1), and homozygous AA (n=77). Median age of donors was 9.6 years (range 0-33.7), counting the donor's age for isolated CBT as 0. The stem cell sources are shown in Table 2. All 234 consecutive patients were transplanted with an MSD following myeloablative CR using busulfan, cyclophosphamide at 200 mg/kg and rabbit ATG at different doses (Table 2 and Figure 1B).

Chimerism was studied by analyzing various polymorphisms after polymerase chain reaction (PCR) amplification of DNA obtained from whole blood at 1, 3, 6, 9 and 12 months after transplantation and every year thereafter. Real-time (RT) quantitative PCR of insertion/deletion polymorphisms was used when the minority chimeric fraction was below 10% and short-tandem-repeat (STR)-PCR was performed when the minority fraction was over 10%. In both cases, analyses were performed according to the kit manufacturer's recommendations (Promega; PowerPlex 16S assay for STR-PCR) and GenDex for Indel RT-PCR (KMRDX Chimerism Assay). When possible, peripheral-blood CD3<sup>+</sup> T cells and CD3<sup>+</sup> cells were selected for analysis.

Exact Fisher tests were used to compare proportions and Wilcoxon rank sum tests for continuous distributions. Patients were censored on the date of death or last visit for Kaplan-Meier (KM) estimates of survival and on the date of event (death, non-engraftment or rejection) or last visit for KM estimates of EFS. Cumulative incidences of rejections, deaths, acute GvHD (aGvHD) grade  $\geq$ II and cGvHD were also estimated by the KM method because of the small number of deaths and rejections. Failure time data curves were compared by the Log rank test. Risk factors for EFS, acGvHD and cGvHD, were analyzed by Cox regression with estimated hazards ratio (HR) and 95% Confidence Interval (CI). Type 1 error was fixed at the 5% level. All tests were two-tailed. Univariate models were fitted and all variables associated with the outcome at the 10% level were retained for introduction into a multivariate model. Statistical analyses were performed on SPSS version 22, and MedCalc (Belgium).

## Results

The median (range) follow up was of 7.9 years (0.1-27.6

**Table 1.** Characteristics of sickle cell anemia patients transplanted in France with matched-sibling donors and myeloablative conditioning regimen.

Period	Nov 1988-Dec 2004 Blood Paper 2007	Jan 2005- Dec 2012 New cohort	Nov 1988-Dec 2012 Overall cohort
<b>PATIENTS' CHARACTERISTICS</b>			
Number	83	151	234
Phenotype, n	81 SS, 2 Sb0	147 SS, 2 Sb0, 2 SDPunjab	228 SS, 4 Sb0, 2 SDPunjab
Sex (F/M)	37/46	73/78	109/125
Median age (range) years	8.8 (2.2-22)	8.1y (3-28.9)	8.4y (2.2-28.9)
Age > 15 years, n (%)	9 (10.8%)	23 (15.2%)	32 (13.7%)
Transfusion number: median (range)	15 (2-119)	16.5 (2-108)	16 (2-61)
Ferritin: median (range) ng/ml	462 (13-3820)	970 (8-6208)	804 (8-6208)
<b>Pre-transplant SCA-complications</b>			
Stroke + TIA, n (%)	30 + 3 (39.8%)	20 + 3 (15.2%)	50 + 6 (23.9%)
Ischemic lesions, n (%)	48 (57.8%)	57 (37.7%)	105 (44.9%)
Silent infarcts, n (%)	16 (19.3%)	34 (22.5%)	50 (21.4%)
Stenoses, n (%)	30 (36.1%)	44 (29.1%)	74 (31.6%)
Moya, n (%)	9 (10.8%)	7 (4.6%)	16 (6.8%)
History of abnormal TCD, n (%)	8 (9.6%)	57 (37.7%)	65 (27.8%)
Abnormal TCD at transplant, n (%)	10 (12.0%)	8 (5.3%)	18 (7.7%)
≥ 3 VOC per year, n (%)	28 (33.7%)	46 (30.5%)	74 (31.6%)
≥ 2 ACS, n (%)	13 (15.7%)	36 (23.8%)	49 (20.9%)
Multiple osteonecroses, n (%)	13 (15.7%)	5 (3.3%)	18 (7.7%)
Red cell alloimmunization (≥ 2), n (%)	2 (2.4%)	5 (3.3%)	7 (3.0%)
Rare erythroid group, n (%)	3 (3.6%)	3 (2.0%)	6 (2.6%)
TRJV >= 2.7m/s, n (%)	0 (0%)	5 (3.3%)	5 (2.1%)
Severe anemia, n (%)	10 (12.0%)	7 (4.6%)	17 (7.3%)
Priapism, n (%)		5 (3.3%)	5 (2.1%)
Splenectomized total/partial, n (%)	4 (4.8%)/0	12 (7.6%)/6 (4.0%)	16 (6.8%)/6 (2.6%)
Seizures, n (%)	7 (8.4%)	4 (2.6%)	11 (4.7%)

Cytomegalovirus status in recipient at transplant was available in 219 of these patients and was positive in 167 (76%). Exclusion criteria were not defined in the protocol, but patients were always discussed in multidisciplinary meetings, and the final decision was left to the physician in charge of the patient. Patients with severe sequelae post-stroke were excluded, but several patients, for example those with hemiparesis, were transplanted. The main issue was to evaluate if the child could withstand the 4-6 weeks hospitalization in the transplant unit.

years). All survivors had at least five years of follow up and results are presented in Table 2.

### Engraftment

Two non-engraftments were observed, with rapid autologous reconstitution, in two patients transplanted with cord blood (CB) (Table 3). The first one, a patient who was transplanted 20 years ago, has not experienced any further SCA-related crisis since transplant, but still has 21% fetal Hb (HbF) (*vs.* <2% before SCT) and was still anemic (Hb 7 g/dL) at last visit. The second one, transplanted 12 years ago, is currently on HU therapy because of recurrent crises. For the other 232 patients, the time to absolute neutrophil count  $>0.5 \times 10^9/L$  was significantly shorter after bone marrow transplantation (BMT) compared to CB transplantation (CBT) [mean±Standard Deviation (SD); 20.7±5.7 *vs.* 32.0±10.0, respectively;  $P<0.001$ ]. Similarly, platelets reached  $50 \times 10^9/L$  sooner after BMT (day 26.5±12.2) than after CBT (day 44.6±18.3;  $P=0.001$ ).

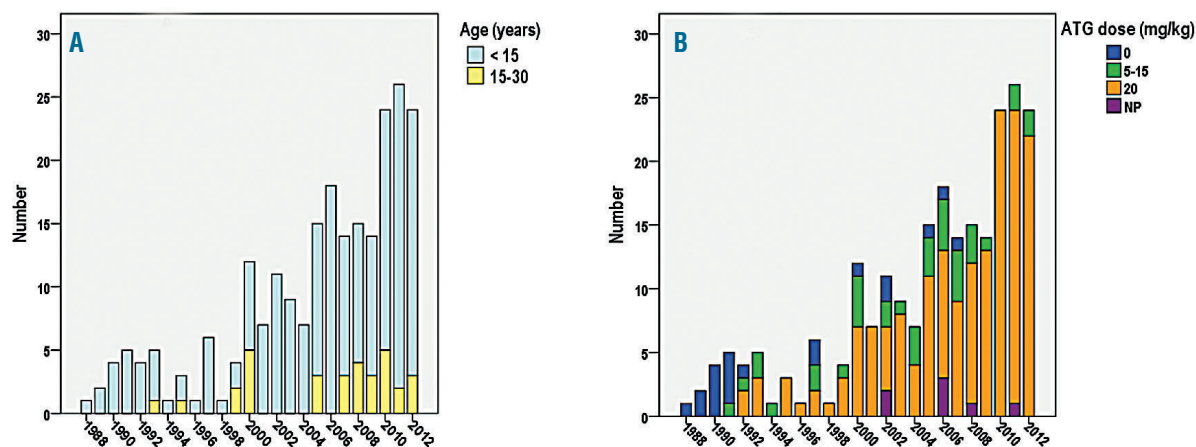
### Rejection

Rejection was defined as donor chimerism <5%. Despite initial successful donor engraftment, six rejections

were observed 0.5, 0.9, 1.2, 2.0, 2.3 and 9-years post transplant in patients transplanted before year 2005, as previously reported.<sup>22</sup> Five of them did not receive ATG as part of the preparative CR, and only one rejection occurred despite CR including ATG. Taking into account the two patients with non-engraftment and the six patients with rejection, the cumulative incidence of rejection for the overall cohort was 3.1% (95%CI: 0.7-5.5%) at five years; this was 20.0% (95%CI: 3.0-37.0%) in patients not prepared with ATG *versus* only 1.4% (95%CI: 0.0-3.0%) in those who received ATG ( $P<0.001$ ). However, the ATG dose had no impact on the rejection risk (Figure 2A).

### Transplant-related mortality

Seven deaths occurred after MSD-SCT. Five occurred in patients transplanted before year 2005, as previously reported.<sup>22</sup> Two deaths occurred at 0.5 and 2.5 years post transplant in the second cohort, because of adenoviral encephalitis<sup>30</sup> and GvHD-related obliterans bronchiolitis, respectively. The cumulative incidence of TRM at five years was 3.0% (95%CI: 0.8-5.2%) and significantly decreased with time from 6.0% (95%CI: 0.8-11.2%) before January 2005 to only 1.4% (95%CI: 0-3.4%) for the 151 patients transplanted since January 2005 ( $P=0.045$ ).



**Figure 1. Matched-sibling donor hematopoietic stem cell transplantation (MSD-SCT) performed in France (1988-2012) after myeloablative conditioning regimen (n=234).** Only 2% of patients were not prepared with anti-thymocyte globulin (ATG) in the second cohort versus 20.5% in the first cohort. The transplantation procedure and prophylaxis for graft-versus-host disease (GvHD) were as previously reported,<sup>22</sup> except that busulfan has been administered intravenously since year 2001 (Busilvex® Pierre Fabre Médicaments, Boulogne-Billancourt, France) from day -10 to day -7 at the total dose of 12.8 mg/kg for patients weighing >34 kg, 15.2 mg/kg for patients weighing 23-34 kg, 17.6 mg/kg for patients weighing 16-23 kg, or 19.2 mg/kg for patients weighing 9-16 kg. Busulfan pharmacokinetics were not performed for the majority of patients (n=202). In addition, cyclosporine was replaced by mycophenolate mofetil after 2002 in case of GvHD requiring steroid therapy. (A) Proportion of patients younger or older than 15 years. (B) ATG doses. NP: not precise; these patients received ATG, but the exact dose was not recorded.

There was no significant difference in TRM ( $P=0.490$ ) between those prepared with 5-15 mg/kg ATG (5.5%; 95%CI: 0-13.1%) and those prepared with 20 mg/kg ATG (3.0%; 95%CI: 0.4-5.6%).

#### Event-free survival

Considering deaths (n=7), non-engraftments (n=2), and rejections (n=5) as events, the overall 5-year EFS was 93.9% (95%CI: 90.7-97.1%), but EFS strongly improved with time since it was 97.9% (95%CI: 95.5-100%) in the 151 patients of the second cohort versus 86.9% (79.5-94.3%) in the 83 patients of the first series (Figure 2B). Among the 190 patients transplanted after year 2000 and prepared with ATG, 5-year EFS was 97.8% (95%CI: 95.6-100%). EFS was similar in patients prepared with 5-15 mg/kg or with 20 mg/kg ATG (Figure 2C), in patients younger or older than 15 years (Figure 2D), and in patients transplanted from CB alone versus BM (Table 3). Cox regression analysis showed that EFS was not associated with the recipient's or donor's age or with the cell source, but was significantly associated ( $P<0.001$ ) with the period of transplant (i.e. before or after year 2000) (HR=11.3; 95%CI: 3.9-33.4).

#### Graft-versus-host disease

Acute GvHD was assessable in the 232 successfully engrafted patients. The overall cumulative incidence of aGvHD  $\geq$ II at day 100 was 20.1% (95%CI: 14.9-25.3%). The multivariate Cox regression analysis retained sex mismatch (HR=2.12, 95%CI: 1.15-3.90;  $P=0.016$ ) and donor's cytomegalovirus (CMV) positive status (HR=5.10, 95%CI: 2.15-12.05;  $P<0.001$ ) as significant and independent risk factors for aGvHD  $\geq$ II (Figure 3A).

Chronic GvHD was assessable in 228 patients, and occurred in 24 patients. It was mostly mild in 18 patients, but extensive in six. Organ involvement included resolutive cutaneo-digestive (n=2), and obliterans bronchiolitis (n=4) which was responsible for death in two patients.

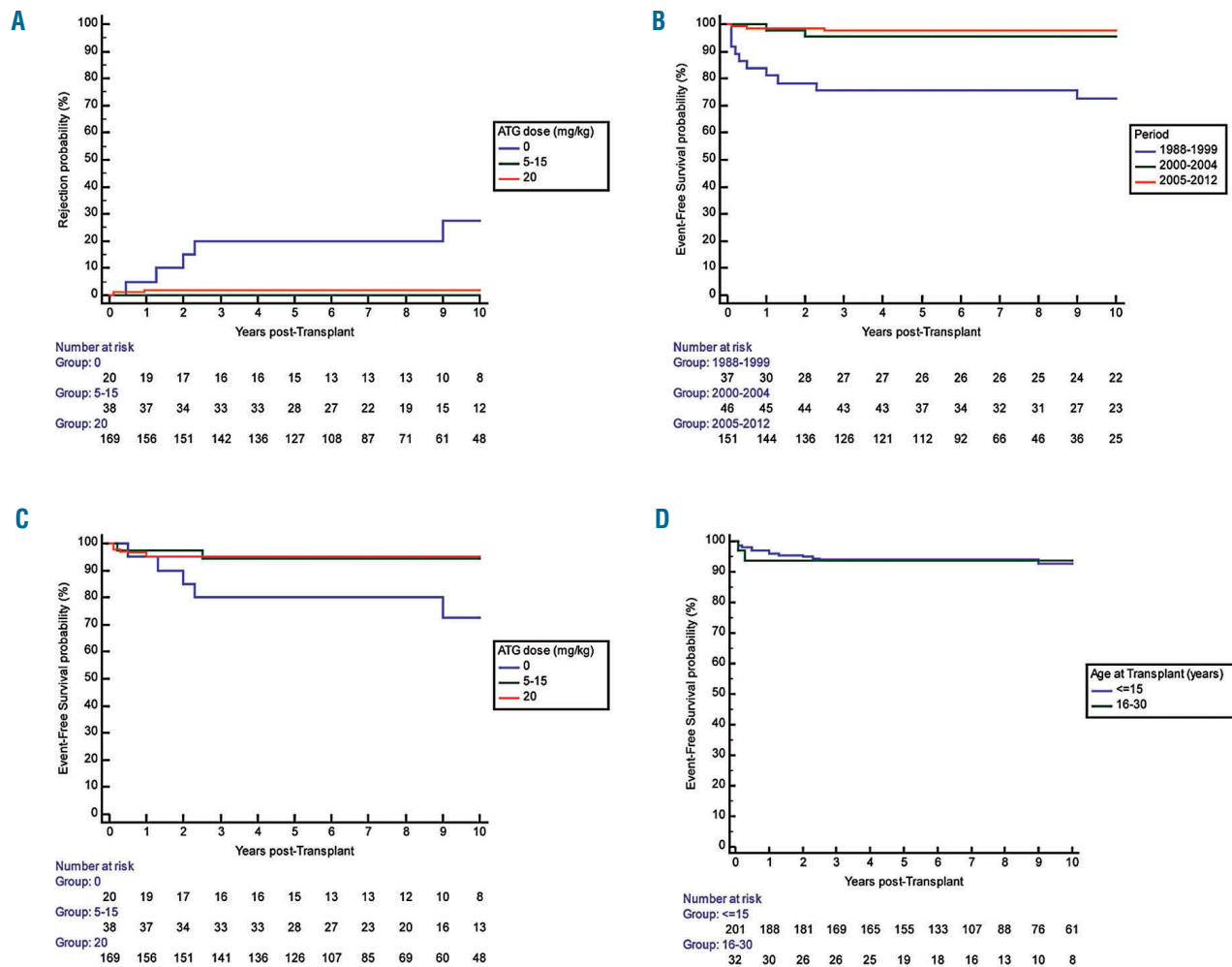
The cumulative incidence of cGvHD at five years was 10.5% (95%CI: 6.5-14.5%), and was significantly lower (5.4%, 95%CI: 1.8-9.0%) in patients who received the highest ATG dose (20 mg/kg) than in those prepared without ATG (25%, 95%CI: 5.6-44.4%) and receiving lower doses (5-15 mg/kg) (27.0%, 95%CI: 12.4-41.6%) (Log Rank  $P<0.001$ ). Moreover, 5-year chronic GvHD in children under 15 years of age was 7.6% (95%CI: 3.8-11.4%) versus 29.7% (95%CI: 13.1-46.3%) in those over 15 years of age. Multivariate Cox regression analysis retained only the recipient's age (HR=1.098 per 1 year increase, 95%CI: 1.033-1.168;  $P=0.003$ ) and the ATG dose (HR=0.91 per mg/kg increase, 95%CI: 0.86-0.96;  $P=0.001$ ) as independent risk factors.

#### Other post-transplant complications

The number of seizures, hemorrhagic cystitis, and veno-occlusive disease are shown in Table 2. The proportion of patients experiencing seizures and/or posterior reversible leukoencephalopathy was significantly reduced in the second cohort (5.3% vs. 18.1%;  $P=0.002$ ). CMV replication occurred in 23.9% of patients. No CMV-related disease occurred with pre-emptive therapy. Epstein-Barr virus (EBV) asymptomatic viral replications had not been systematically assessed in the first cohort, and were observed in 15 patients (6%) in the second cohort. Six of them required anti-CD20 treatment, but none developed post-transplant lymphoproliferative disease. Among the 234 patients, B-lymphoma occurred six years post transplant in one patient who had received long-term immunosuppressive therapy, but no other secondary malignancies were observed.

#### Gonadal function

All females who were post-pubertal at the time of transplantation (n=14) developed amenorrhea with low serum estradiol and elevated LH and FSH levels during the year following transplant, necessitating hormone replacement



**Figure 2. Rejection probability and event-free-survival (EFS) in sickle cell anemia (SCA) patients transplanted with matched-sibling donor (MSD) after myeloablative conditioning regimen.** (A) Probability of rejection according to the anti-thymocyte globulin (ATG) dose. Overall cumulative incidence of rejection at five years was 20.0% (95%CI: 3.0-37.0%) in patients prepared without ATG and only 1.4% (95%CI: 0.0-3.0%) ( $P<0.001$ ) in those prepared with ATG. However, the risk of rejection was not associated with the ATG dose (5-15 mg/kg vs. 20 mg/kg). (B) EFS according to the ATG dose. EFS was similar after bone marrow transplantation (BMT) and cord blood transplantation (CBT), and in patients prepared with 5-15 mg/kg ATG than in those prepared with 20 mg/kg. (C) EFS in 234 patients depending on the period of transplant. EFS improved strongly as it was only 73.3% (95%CI: 58.7-87.9%) among the 38 patients transplanted before year 2000 and 97.4% (95%CI: 95.0-99.8%) in the 196 patients transplanted after year 2000. (D) EFS according to age over or under 15 years. EFS was similar in patients younger or older than 15 at transplant.

therapy. Thirty-two girls were pre-pubertal at transplantation, and most required hormonal therapy to develop secondary sexual characteristics at the bone age of 13 years. However, 9 of 32 of the pre-pubertal girls at SCT, who had reported to have spontaneously undergone normal puberty at the last visit, were significantly younger at transplant than those who required hormonal substitution for puberty induction [mean (SD) age 5.9 (2.6) vs. 10.1 (2.1);  $P=0.002$ ]. At last visit, 20 females were older than 25, and four of them, who had been transplanted between 1988 and 1998 at 5.8, 6.1, 6.6 and 7.7 years of age, respectively, had had six spontaneous pregnancies and five children at approximately 20 years after SCT. Pre-transplant ovarian tissue cryopreservation has been systematically performed since year 1998. Unilateral oophorectomy was performed by laparoscopy under general anesthesia and ovarian cortical fragments were cryopreserved at a median age of 8.0 years (range: 3.0-26.4). Among the 93 girls trans-

planted since 1998, 11 were older than 25 years at the last visit; two wanted to become pregnant and requested autograft of ovarian fragments. The first of these was transplanted at 20 years of age and had received hormone replacement therapy because of post-transplant ovarian failure. Orthotopic ovarian fragment autograft was performed by laparoscopy 29 months after SCT, and the first signs of recovery of ovarian function were observed nine weeks after ovarian graft. Hormone replacement therapy was stopped at four months and the patient became pregnant six months after ovarian autograft, delivering a healthy girl at 38 weeks of gestation. She had another child three years later without requiring a new graft of ovarian fragments.<sup>31</sup> In the second patient, transplanted at 22 years of age, autograft of ovarian fragments was performed 12 years post SCT, and recovery of ovarian function was observed after four months, but unfortunately no pregnancy occurred thereafter.

**Table 2.** Matched-sibling donor hematopoietic stem cell transplantation (MSD-SCT) procedure and outcome in sickle cell anemia patients transplanted in France.

Period	Nov 1988-Dec 2004 Blood Paper 2007	Jan 2005- Dec 2012 New cohort	Nov 1988-Dec 2012 Overall cohort
Number of Patients	83	151	234
Conditioning regimen	Busulfan/Cyclophosphamide		
ATG dose	Rabbit ATG		
0 mg/kg, n (%)	17 (20.5%)	3 (2.0%)	20 (8.5%)
5-15 mg/kg, n (%)	18 (21.7%)	18 (11.9%)	36 (15.4%)
20 mg/kg, n (%)	40 (48.2%)	124 (82.1%)	164 (70.1%)
not recorded, n (%)	8 (9.6%)	6 (4.0%)	14 (6.0%)
Stem cell source			
Bone Marrow (BM), n (%)	70 (84.3%)	125 (82.8%)	195 (83.3%)
Cord Blood (CB), n (%)	11 (13.3%)	19 (12.6%)	30 (12.8%)
CB+BM, n (%)	1 (1.2%)	7 (4.6%)	8 (3.4%)
PBC, n (%)	1 (1.2%)	0 (0%)	1 (0.4%)
Outcome			
Median Follow-up (range)	13.8 years (0.1-27.6)	6.8 years (0.4-13.2)	7.9 years (0.1-27.6)
Non-Engraftment, n (%)	1 (1.2%)	1 (0.7%)	2 (0.9%)
Rejection, n (%)	6 (7.2%)	0 (0%)	6 (2.6%)
Deaths, n (%)	5 (6.0%)	2 (1.3%)	7 (3.0%)
Acute GvHD, grade (n)	II (n=11), III (n=4), IV (n=2)	II (n=24), III (n=5), IV (n=1)	II (n=35), III (n=9), IV (n=3)
Chronic GvHD,	mild (n=7), extensive (n=2)	mild (n=11), extensive (n=4)	mild (n=18), extensive (n=6)
Cumulative Incidences (95%CI)			
Non-Engraftment/Rejection at 5 years	7.5% (1.5-13.5%)	0.7% (0-2.1%)	3.1% (0.7-5.5%)
Transplant Related Mortality at 5 years	6.0% (0.8-11.2%)	1.4% (0-3.4%)	3.0% (0.8-5.2%)
Event-Free Survival at 5 years	86.9% (79.5-94.3%)	97.9% (95.5-100%)	93.9% (90.7-97.1%)
Acute GVHD $\geq$ II at Day 100	20.4% (11.6-29.2%)	20.0% (13.4-26.6%)	20.1% (14.9-25.3%)
Chronic GVHD at 5 years	11.2% (4.2-18.2%)	10.1% (5.1-15.1%)	10.5% (6.5-14.5%)
Other post-SCT complications			
Seizures, n (%)	15 (18.1%)	1 (0.7%)	16 (6.8%)
Hemorrhagic cystitis, n (%)	6 (7.2%)	8 (5.3%)	14 (6.0%)
Veno-occlusive disease, n (%)	0 (0%)	2 (1.3%)	2 (0.9%)
CMV replication	18 (21.7%)	38 (25.2%)	56 (23.9%)

Cytomegalovirus (CMV) donor status, available in 217, was positive in 133 patients (61%). ABO compatibility was available in 212 donor-recipient couples, and major and minor incompatibility was present in 34 (16%) and 36 patients (17%), respectively. There was donor/recipient sex mismatch in 120 cases (FM: 61; MF 59).

All the boys who were pre-pubertal at transplant and of pubertal age at the last visit developed normal puberty with normal testosterone, FSH and LH levels, in keeping with their bone age and pubertal status. At last visit, 19 males were over 25 years of age. Three of them who had been transplanted 11, 12 and 20 years ago fathered spontaneously. Pre-transplant sperm cryopreservation was performed in all pubertal males and pre-transplant testicular tissue cryopreservation has been systematically performed since year 2010 in prepubertal boys (n=25) (median age: 7.5 years; range: 3.4-10.2). However, so far, no patient has requested testicular fragment autografting (median age at last visit: 13.7 years; range: 5.8-21.2).

### Chimerism studies

Chimerism was assessable in 208 patients and was categorized as full donor chimerism (>95% donor cells), rejection (<5% donor cells), and low (5-50% donor cells) or high (50-95% donor cells) mixed chimerism. At one year, full donor chimerism was present in 112 patients

(54%), while 92 patients (44%) had mixed chimerism (83 high, and 9 low), and 4 (2%) had <5% donor cells (the 2 patients with no-engraftment and 2 who rejected the graft at 0.5 and 0.9 years).

Multivariate logistic regression analysis showed that the ATG dose (OR=1.06 per 1 mg/kg increase, 95%CI:1.01-1.12;  $P=0.017$ ) and recipient's age (OR=0.94 per year increase, 95%CI: 0.88-0.99;  $P=0.041$ ) were independently inversely associated with the presence of mixed chimerism at one year. Moreover, cGvHD was significantly more frequent ( $P=0.018$ ) in patients with full donor chimerism (18 of 112; 16.1%) than in those with mixed chimerism (5 of 92; 5.5%). These five patients all had donor cell% >75%. No significant association was found between ABO compatibility and chimerism.

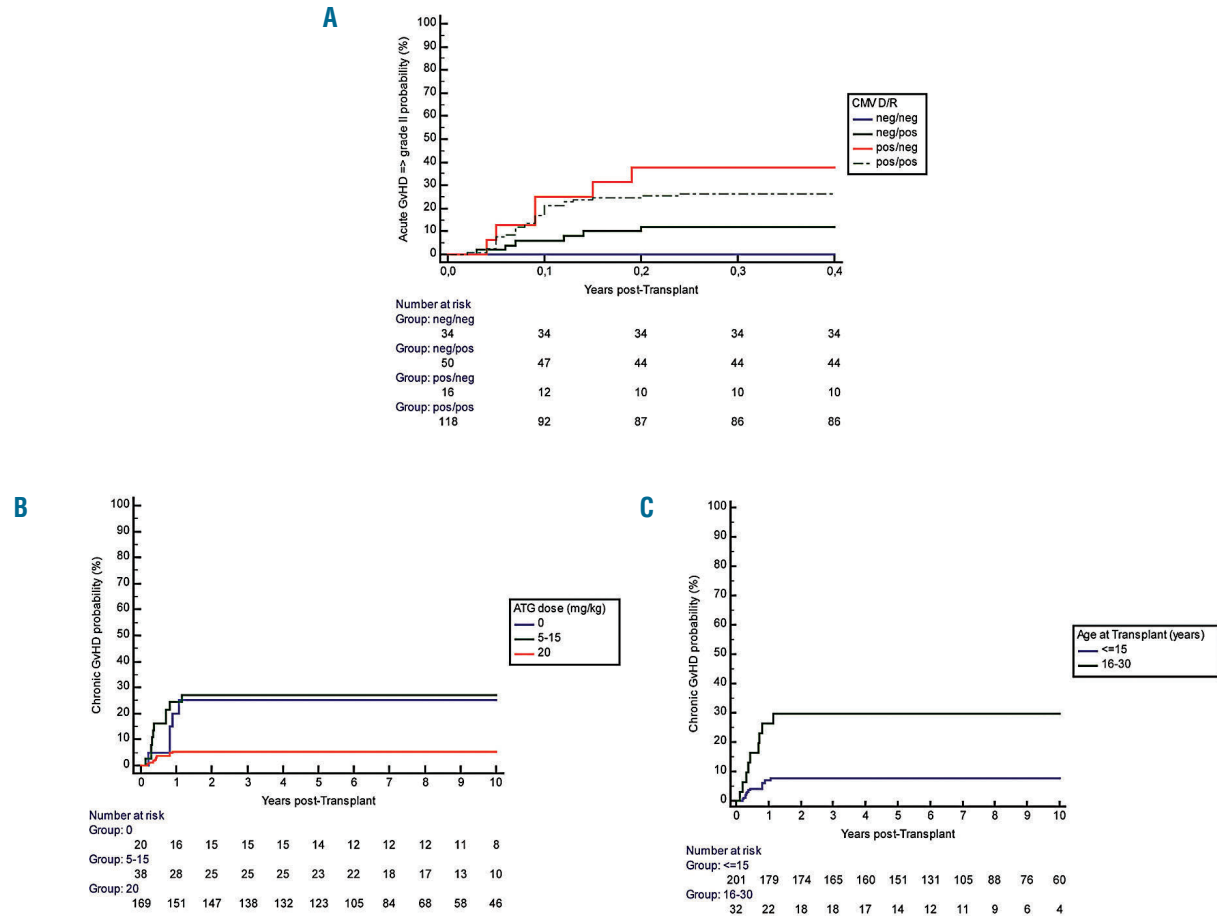
Chimerism on separated populations (CD3<sup>+</sup> and CD3<sup>-</sup>) was assessed in 103 patients. Whole blood donor cell chimerism was more significantly correlated with the CD3<sup>-</sup> ( $r=0.917$ ,  $P<1\times 10^{-12}$ ) than with the CD3<sup>+</sup> ( $r=0.418$ ,  $P<1\times 10^{-15}$ ) population. During the first two years post

transplant, CD3<sup>+</sup> chimerism was significantly lower than CD3<sup>+</sup> and whole blood donor cell chimerism, but was similar thereafter.

**Long-term outcome of chimerism**

One-hundred and twelve patients remained stable with donor chimerism >70% at last visit. However, one girl with a rare erythroid group (U-), transplanted in 2000

from her brother's CB (U+), had full donor chimerism at one and two years that remained stable for five years, but decreased thereafter with the appearance of hemolytic anemia. The patient was given donor lymphocyte infusions (DLI), which allowed the reversion to full donor chimerism along with anemia and hemolysis correction, but it induced cGvHD which was responsible for chronic pulmonary disease (currently well under control).



**Figure 3. Relationship of graft-versus-host disease (GvHD) and donor/recipient (D/R) cytomegalovirus (CMV) status, anti-thymocyte globulin (ATG) dose and recipient age.** (A) Acute GvHD according to the D/R CMV status. Cumulative incidence of acute-GvHD at day 100 according to the D/R CMV status. (B) Chronic GvHD according to the ATG dose. Cumulative incidence of chronic GvHD at five years was significantly lower in the patients having received the high dose of ATG (20 mg/kg) (5.4%, 95%CI: 1.8-9.0%) versus those not prepared with ATG (25%, 95%CI: 5.6-44.4%) and those having received lower doses (5-15 mg/kg): 27.0%, 95%CI: 12.4-41.6%) (Log Rank *P*<0.001). (C) Chronic graft-versus-host disease (GvHD) according to recipient age at transplant. Cumulative incidence of chronic GvHD in children under 15 years of age was 7.6% (95%CI: 3.8-11.4%) versus 29.7% (95%CI: 13.1-46.3%) in those older than 15.

**Table 3. Rejection, transplant-related mortality, event-free survival as a function of the cell source, bone marrow (BM) versus cord blood (CB) alone.**

	CB alone (n=30)	BM (n=195)	P
Non-engraftment (n)	2	0	0.017
Late rejection	0	6	NS
Cumulative Incidence of rejection at 5 years (including non-engraftment)	6.7% ± 9.2%	2.7% ± 2.4%	NS
Deaths	0	7	NS
Events	2	13	NS
EFS at 5 years	93.3% ± 9.2%	93.7% ± 3.6%	NS
Cumulative Incidence of acute GVHD ≥ grade II	13.3% ± 12.4%	21.1% ± 5.8%	NS
Cumulative Incidence of chronic-GVHD	3.3% ± 6.6%	11% ± 4.6%	NS
EFS chronic-GVHD free at 5 years	90.0% ± 10.1%	84.5% ± 5.2%	NS

Among the 92 patients with mixed chimerism at one year, four rejected the graft (4.3%) at 1.5, 2, 2.3 and 9 years post transplant, but none had been prepared with ATG. Among the other 88 patients, none had rejected the graft at last visit, nine had switched to full donor chimerism, and 69 had high and 10 low mixed chimerism (Table 4). None of those with low mixed chimerism experienced SCA-related vaso-occlusive crisis (VOC) or acute-chest-syndrome (ACS), but five had hemoglobin <100 g/L or reticulocytes >150x10<sup>9</sup>/L, considered as SCA-related symptoms. Thus, despite the absence of rejection and the absence of VOC or ACS occurrence, these patients were deemed as having SCA recurrence, resulting in an overall disease-free survival (DFS) of 95.5% at five years (95%CI: 92.7-98.3%). Three of the patients with low mixed chimerism and hemolytic anemia stigmata received DLI, which did not induce GvHD, but did not offer improvement of anemia and hemolysis. Figure 4 reports all biological data recorded at the same time as chimerism during the entire follow up. It can be seen that as long as donor chimerism remains higher than 50%, Hb and reticulocytes remain normal, resulting in no anemia, no hemolysis and similar HbS% as donor.

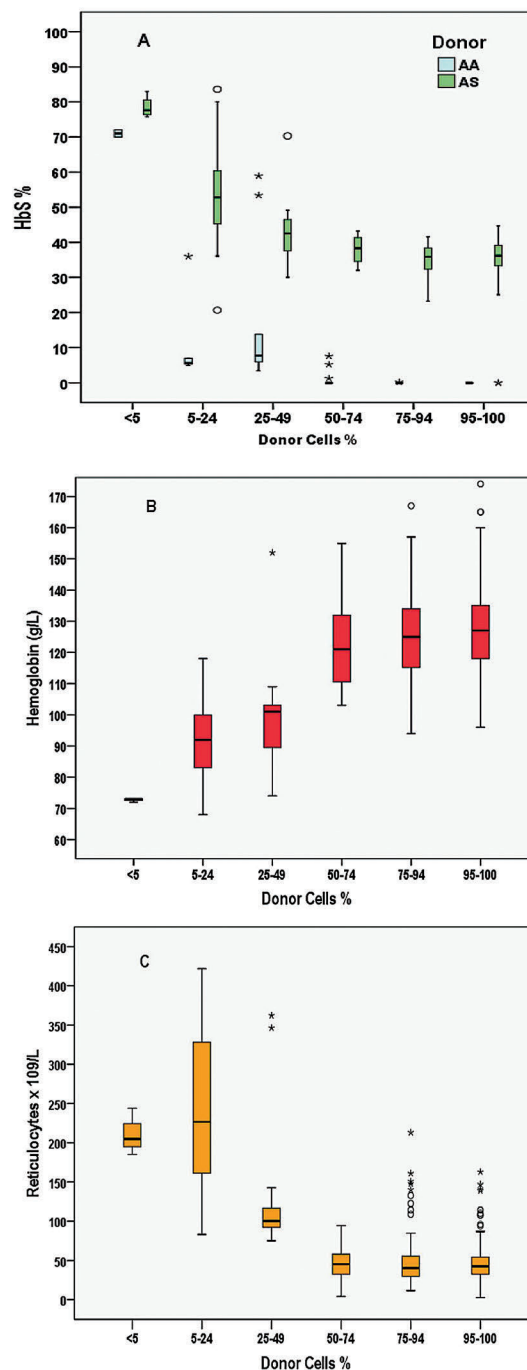
## Discussion

In this report, we confirm that SCA children and young adults transplanted after year 2000 (n=197) with an MSD and prepared with myeloablative-CR associating busulfan, cyclophosphamide and ATG have at least a 95% chance of cure. These results compare favorably with other published series using myeloablative<sup>19,26</sup> and non-myeloablative<sup>27-29</sup> CR.

The TRM < 2% in this series must be interpreted in the context of mortality risk related to the SCA disease itself, which was reported to be 6.1% before the age of 18 years in the Dallas cohort,<sup>16</sup> and 1% in the London<sup>17</sup> and 2.5% in the Créteil<sup>14</sup> newborn cohorts. Thus, MSD-SCT does not expose children to a higher risk of death than SCA itself, but offers improved quality of life. Moreover, it is important to remember that transition to adulthood carries a high risk of death,<sup>32</sup> and that mortality in adults<sup>18</sup> has increased during the last few years, whereas it has significantly decreased for children.

Except for frequent high fever during ATG infusion, myeloablative conditioning was well tolerated in the 234 young patients, with only one death during aplasia and two mild veno-occlusive diseases. High doses of ATG are known to increase the risk of viral complications. The occurrence of EBV reactivation, however well controlled with anti-CD20 treatment, and the case of fatal adenoviral meningoencephalitis<sup>30</sup> are of concern, but the risk benefits during viral infections, which are manageable, and cGvHD, which is more difficult to control, need to be taken into account. Seizures and posterior reversible encephalopathy syndromes (PRES), which were highly frequent (16 of 83; 19%) before year 2005,<sup>22</sup> despite preventive measures such as anticonvulsant prophylaxis with clonazepam during busulfan administration and cyclosporine therapy, strict control of arterial hypertension, prompt correction of magnesium deficiency, maintenance of the Hb concentration above 9 g/dL and platelet count above 50x10<sup>9</sup>/L,<sup>33</sup> were significantly ( $P=0.002$ ) less frequent after 2005 (8 of 151; 5%), following the SFGM-

TC recommendation to promptly replace cyclosporine with mycophenolate mofetil in case of GvHD requiring steroid therapy.<sup>22</sup> However, the risk of seizures and PRES remains an adverse effect of cyclosporine and steroid therapy,<sup>34</sup> and may warrant substituting cyclosporine for



**Figure 4.** Relationship between % donor cells and hemoglobin S percentage (HbS%), hemoglobin (Hb) level, and reticulocyte count. All biological data (HbS%, Hb and reticulocytes) recorded at the same time as the chimerism during the overall follow up were used for these box-plots. (A) Box-plot of HbS% according to the donor cell% category. This graph shows that HbS% is similar to that of the donor (AA or AS) as long as donor cell% is higher than 50%. (B) Box plot of Hb level according to donor cell% category. Hb level remains higher than 100g/L as long as donor cell% is higher than 50%. (C) Box plot of reticulocyte count according to % donor cells. Reticulocyte count remains lower than 100x10<sup>9</sup>/L as long as % donor cells is higher than 50%.



sirolimus<sup>27,28</sup> that is less neurotoxic and induces good tolerance.<sup>35</sup> Hemorrhagic cystitis occurred in 21 of 234 patients (9%) and was particularly severe in two patients. Cyclophosphamide is generally considered to be the most important predisposing factor for occurrence of hemorrhagic cystitis.<sup>36,37</sup> Despite the absence of prospective randomized trials comparing busulfan-fludarabine to busulfan-cyclophosphamide, several reports mention a lower risk of hemorrhagic cystitis with fludarabine.<sup>38,39</sup> This complication may suggest replacing cyclophosphamide with fludarabine, as only a 2% risk of hemorrhagic cystitis was reported in children transplanted for non-malignant disease following busulfan-fludarabine *versus* 9% with busulfan-cyclophosphamide CR.<sup>39</sup>

The major long-term concern with busulfan is the ovarian failure observed in post-pubertal females and infertility risk in both genders.<sup>40,41</sup> Despite the occurrence of spontaneous puberty in girls transplanted at a younger age and the birth without treatment of several babies from females transplanted 20-25 years ago during infancy, demonstrating some reversibility of ovarian dysfunction, it remains crucial to maximize the chances of fertility and to recommend pre-transplant cryopreservation of ovarian<sup>42-44</sup> and testis<sup>45</sup> tissues before SCT with myeloablative CR. In France, systematic ovarian and testicular tissue cryopreservation has been established since 1998 and 2010, respectively, and is free of charge. However, CR preserving fertility such as the one proposed by the National Institutes of Health (NIH) protocol using 3Gy total body irradiation with testis shielding in males and alemtuzumab<sup>27,29</sup> should be preferred when gonadal cryopreservation is not easily accessible and for adults. It is also important to keep in mind that fertility is also compromised by SCA itself,<sup>46</sup> and that this risk is lower when SCT is performed at a younger age.

Event-free survival in this series was similar for patients younger and older than 15 years, but the risk of cGvHD, not acceptable in this non-malignant disease, was much more frequent in older patients. We show that the high dose of ATG (20 mg/kg) significantly reduced the risk of cGvHD independently of the age at transplant by favoring mixed chimerism occurrence. In the 20 of 234 patients not prepared with ATG, mixed chimerism was unstable, and secondary graft failed in 5 of 20. In contrast, only one secondary rejection was observed before year-1 among the

214 patients prepared with ATG.

Mixed chimerism was present in 44% of patients at one year in this series. None of the patients prepared with ATG rejected the graft and none experienced VOC or acute chest syndrome. The absence of SCA-related symptoms in patients with mixed chimerism has been reported in other series.<sup>22,47-50</sup> Low levels of donor erythroid engraftment were shown to result in donor cell predominance among erythroblasts and erythrocytes.<sup>48</sup> This stimulated the development of non-myeloablative CR in order to reduce TRM and preserve fertility, and resulted in the recommendation of HSCT to older adult patients with organ dysfunction. While the first results after non-myeloablative SCT have been disappointing, very encouraging results were obtained by the NIH team in adults (17-64 years of age)<sup>27,28</sup> using as stem cell source granulocyte-colony stimulating factor (G-CSF)-stimulated peripheral blood progenitors from HLA-matched sibling donors and sirolimus for GvHD prophylaxis, which was stopped after one year in those with >50% donor T-cell chimerism. The mean duration of immunosuppression was 2.1 years (range: 1.0-8.4). Among 30 patients transplanted, four experienced rejection (13%), which was responsible for one death in a patient with Moya Moya, but no TRM and no GvHD were observed. Mean donor T cell was 48%, mean myeloid chimerism was 86%, and 15 patients had stable chimerism despite sirolimus withdrawal. These results were reproduced in 13 adult patients by another team in Chicago.<sup>29</sup> Thus, among the 43 patients reported in both series, 88% were alive, free of SCA and without GvHD. However, the limitations of the protocol are the long-term immunosuppression and the 300 cGy TBI that could favor a secondary malignancy. Other non-myeloablative or reduced intensity CR have been reported by several teams and fully described in a review,<sup>50</sup> but the most successful results were obtained with the NIH protocol described above.

It is to be noted that the excellent results reported here mostly concern young patients and are less applicable to older patients with higher morbidities. Moreover, outcomes of cognitive performances, quality of life, costs and organ functioning are not reported here. This is an important limitation of our study but only prospective trials comparing MSD-SCT to standard-care would be able to define the true risk benefit balance for each procedure.

**Table 4. Biological data in patients with low mixed chimerism (5-49% donor cells) at last visit.**

Donor genotype	Years post-HSCT	Donor cells % at 1 year	Donor cells % at last visit	HbS %	Hb g/L at last visit	Retic x10 <sup>9</sup> /L
AS	15.5	24	19	51	103	226
AS	5.1	60	20	60	83	230
AS	7.1	35	21	70	69	422
AS	14.1	46	22	63	87	360
AS	15.4	73	42	44	151	79
AA	12.1	20	12	36	73	138
AA	9.5	30	20	30	130	nd
AA	10.2	32	22	5	118	83
AA	6.2	61	30	10	100	101
AA	5.9	57	38	8	103	75

HSCT: hematopoietic stem cell transplantation; HbS: sickle hemoglobin; Retic: reticulocytes.

Furthermore, this study does not allow the chances of fertility after ovarian or testicular reimplantation to be estimated. However, the present study is the first to report the very long-term outcome in patients with mixed chimerism and to show that the correction of hemolytic anemia requires a higher donor cell% than the prevention of SCA-related crises.

The issue now is to determine whether a protocol offering an EFS at 87% with no TRM and no GvHD should also be favored for children. In France, the myeloablative CR with 98% EFS with a very low risk of cGvHD is still currently used in children under 15 years of age, but we recommend the NIH protocol for patients older than 15.

However, we recognize that this is still a matter of debate and additional prospective studies may result in a change in course depending on the long-term results of MSD-SCT with non-myeloablative CR.

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