Long-Term Insulin Independence in Type 1 Diabetes Mellitus Using a Simplified Autologous Stem Cell Transplant

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Context: Efforts to find a cure for type 1 diabetes have focused on the removal of the autoimmune pathophysiologic substrate, with the use of immunosuppressive regimens including Autologous hematopoietic stem cell transplantation (AHSCT).

Objective: The main objective of determining long-term insulin independence as well as changes in glycated hemoglobin (HbA1c). Secondary outcomes were procedure morbidity and the need for hospital management.

Design: We enrolled patients with type 1 diabetes between 2012 and 2014. Median follow-up was 34 months (range 25–56).

Setting: Ambulatory care.

Intervention(s): We decided to carry out an AHSCT protocol using a less toxic and affordable simplified method based on fludarabine in an outpatient setting.

Patients: Patients were of both sexes, aged 8 to 25 years old, with positive levels of anti-GAD antibodies, a C-peptide level >1.0ng/mL and less than 3 months since diagnosis.

Main Outcome Measure(s): Insulin independence.

Results: Sixteen patients were included. Overall response was 81% with seven patients achieving insulin independence (44%); six were partial responders (37%), while three were non-responders (19%). The HbA1c level showed a mean decrease of -2.3% at the 6 months in the insulin independence group. Median age was 12 years (range 8–17). A mean of $11.5x10^6$ CD34+ cells (SD±8.2) was obtained. Related mortality at 100 days was 0% as well as during follow-up. Outpatient setting was 100%.

Conclusions: Simplified AHSCT in an outpatient setting is a feasible, safe and potentially therapeutic intervention for early-onset type 1 diabetes.

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Abbreviations:

Type 1 diabetes is a multifactorial disease that occurs in genetically susceptible subjects and is characterized by an attack on pancreatic islet β cells by antibodies directed at glutamic acid decarboxylase (anti-GAD), insulin, insulinoma-associated autoantigen 2 and zinc transporter 8 (1). The resulting damage reduces the production of insulin until clinical manifestations become apparent (2, 3). Incidence varies considerably from 0.1/100,000 in India to more than 60/100,000 in Finland (4, 5). In Mexico, the number of new cases has increased from 3.4 to 6.2/100,000 among individuals aged $<$ 19 years in the last decade (6, 7).

Pathophysiology is complex and controversial, but functional defects in the immune system and β cells are related to type 1 diabetes (8, 9, 10), and efforts to find a cure have focused on immunosuppression (3, 4, 11). The loss of β cells is hallmarked by the presence of CD8+ T-lymphocytes within the islet lesions (12). There is no explanation for the destruction of β cells but proinsulin is a predominant antigen for the ultimate development of type 1 diabetes (13). Genetic studies of loci associated with the disease have linked its influence to aberrant control of the immune response (14). Insulin replacement does not always confer the metabolic regulation required to prevent complications. As a consequence, preserving insulin secretion has become a therapeutic target and rapid intervention is critical (15). In this setting, autologous hematopoietic stem cell (HSC) transplantation (AHSCT) has been conducted in autoimmune diseases, using the principle of "immunological reset" (16, 17, 18, 19) and type 1 diabetes has been studied using the same principle. More than 7 years ago, Voltarelli et al performed hematopoietic cell transplants in patients with newly diagnosed type 1 diabetes with encouraging results (20, 21). These results were replicated by others (22, 23). However, intensive conditioning regimens using 200 mg/kg of cyclophosphamide plus antithymocyte globulin as employed in these early studies are not without serious risk. As expected, several complications have occurred, including one death (21, 24). A balance between optimal immunosuppression and adverse effects from this kind of intervention is mandatory. Therefore, we decided to carry out a prospective, single center, autologous hematopoietic cell transplant pilot trial in patients with newly diagnosed type 1 diabetes, using a less toxic and simplified method (25, 26).

Materials and Methods

Between May 2010 and July 2013 patients with type 1 diabetes according to the criteria of the American Diabetes Association (ADA) were invited to participate. Inclusion criteria were patients of both sexes, aged 8–25 years and with positive anti-GAD antibodies. Patients with a previous ketoacidotic crisis were also included. Other criteria included a C-peptide level $>$ 1.0 ng/mL either during a 2 hours mixed-meal tolerance test (boost) or during an intravenous (IV) glucose tolerance test (IVGTT) with 25 g of glucose and less than 3 months from diagnosis. The Islet Antigen 2 (IA2) antibodies were evaluated. Exclusion criteria were the presence of infection or another serious disease, pregnancy, glucocorticoid treatment, diabetes duration greater than 3 months, and C-peptide $<$ 1.0 ng/mL 2 hours after a mixed meal tolerance test or after IVGTT. The protocol was approved by the ethics committee at the institution and registered at Clinical Trials.gov (NCT01121029). The parent or legal guardian signed an informed consent before any procedure. The main objective was to determine long-term insulin independence as well changes in glycated hemoglobin (HbA1c). Secondary objectives were to assess procedure morbidity and the need for hospitalization. At six months post-transplant, HbA1c as well as C-peptide, insulin and glucose levels using a mixed-meal tolerance test (baseline, 30 minutes, 60 minutes, 90 minutes, and 120 minutes) were determined in order to register potential changes in patient insulin production. We use HbA1c value for long-term evaluation.

C-peptide levels were measured with a 2-hour mixed-meal tolerance test by electrochemiluminescence with an Elecsys 2010® (Roche diagnostics) analyzer with a CV 1.5%. This test was performed after an 8- to 12-hour fast with administration of 240 ml Boost®, allowing only prandial coverage with ultrarapid insulin, if needed. Determination of glycosylated hemoglobin was performed using high-performance liquid chromatography (HPLC) (D-10 Biorad) standardized to DCCT values; IA-2 titers were measured by radiobinding assay and anti-GAD titers were measured by radioimmunoassay (RIA) (Diagnostic Systems Laboratories Inc, Webster, Texas). All subjects involved were asked to record their daily insulin requirements and pre- and postprandial capillary glucose measures during the transplant period and follow-up. Capillary glucose records were obtained by home self-monitoring and insulin doses were calculated and adjusted using a sliding scale for the prandial doses and the fasting plasma glucose for the basal dose. If diabetes control was out of target, insulin doses were adjusted. Diets during and after transplantation were according to ADA recommendations available at that time (27). Following transplant, and according to glycemic pre- and postprandial levels, an attempt was made to discontinue the use of insulin; first the prandial doses, and then the basal dose. After six months of follow-up, patients were categorized into 3 groups: those achieving complete insulin independence, defined as a total independence of insulin; nonresponse; ie, those achieving $<$ 10% reduction in their total daily insulin requirements; and partial insulin independence; ie, those with a reduction of 11 to 99% of their total daily insulin requirements. Knowing that in the pubertal phase there is a physiological state of insulin resistance (and accordingly, a need for greater doses of insulin), we estimated an "expected dose of insulin based on age" to be 0.7 U/kg/d for patients younger than 11 years (reported values: 0.6–0.9 U/kg/d), and an insulin dose of 1.5 U/kg/d for patients older than 12 years (reported values: 1.0–1.5 U/kg/d) (28).

Autologous HSC transplant (AHSCT)

The procedure was performed at the outpatient transplant clinic. Afterwards, the patients were sent home (no more than 10 km from our clinic) where they remained under the care of a family member previously instructed about diet composition,

hygiene, medication management and signs of emergency. Patients were seen every day until engraftment was achieved.

Mobilization

Autologous stem cells were mobilized using cyclophosphamide, with a total dose of 3.0 g/m^2 administered on 2 consecutive days. After 2 days granulocyte colony stimulating factor (G-CSF) was applied subcutaneously at a dose of 10 μg/kg/d for 6 days or until a CD34+ count $>$ 10 cells/μL in peripheral blood was reached. Patients also received uroprotection by means of mesna 500 mg/m^2 IV during the first two days of mobilization and ondansetron 5 mg/m^2 to prevent nausea. Collection through a peripheral vein was attempted. In those cases where this was not possible, a central venous catheter was used. One or two apheresis procedures were performed using a COBE-Spectra system (Gambro, Lakewood, CO) based on the Spin-Nebraska protocol. The goal of peripheral collection was at least 2.0 $\times$ 10^6 viable CD34+ cells/kg body weight; quantification was performed by flow cytometry.

Conditioning regimen and transplantation

The conditioning regimen consisted of cyclophosphamide 500 mg/m^2/d IV + fludarabine 30 mg/m^2/d PO for 4 days with a total dose of 2 g/m^2 and 120 mg/m^2, respectively. The same dose/kg was used for all ages. This regimen began after apheresis was completed and once the required minimum number of CD34+ cells had been collected. Patients received chemotherapy in the outpatient clinic and were discharged once the procedure had been completed. Ondansetron i.v. was administered daily at a dose of 5 mg/m^2 prior to the application of chemotherapy; later a PO dose of 4 mg every 8 hours was used to maintain the antiemetic effect. Noncryopreserved stem cells were infused 24 hours after the last dose of chemotherapy, this being day 0. Ciprofloxacin (500 mg/bid (twice a day)), fluconazole (100 mg/qd) and acyclovir (400 mg/bid) were prescribed to all patients until grafting had occurred. We defined successful grafting as an absolute neutrophil count $>$ 0.5 $\times$ 10^9/L for at least 3 consecutive days and platelet grafting as a count $>$ 20 $\times$ 10^9/L without platelet transfusion. In order to establish a basis for comparison in evolution, we included a historical control group with newly diagnosed type 1 diabetes patients from the same hospital registry, matched as far as possible to the trial group, according to the same characteristics.

Statistical analysis

For descriptive data, measures of central tendency for all continuous variables are presented. Dual comparisons between quantitative variables were performed using Student´s *t* test to determine normality. For categorical variables, the χ^2 test was used by adjusting the p-values using Fisher and McNemar statistics, as appropriate. By segmentation of the overall curve, the area under the curve (AUC) was calculated for each of the measurements of C- peptide during the mixed-meal tolerance tolerance (baseline, 30, 60, 90 and 120 minutes). We used a multiple regression model of random effects for comparisons between observation periods. The same method was used to contrast measurements of anti-GAD titers. To show changes in HbA_{1c} levels, these were compared using mixed-effects linear regression considering normally distributed residuals. Statistical analysis was performed using SPSS v. 20 (IBM, Armonk, NY) and Microsoft Excel 2010 (Microsoft Inc., Redmond, WA).

Results

Nineteen subjects with type 1 diabetes were studied for potential inclusion in the protocol, and 16 were eventually recruited: 7 women and 9 men. Their baseline characteristics are shown in Table 1. The mean AUC of the C-peptide level was 307.78 (SD $\pm$ 228.43). Baseline measurements of anti-GAD and HbA1c showed means of 9.42 U/mL (SD $\pm$ 13.26) and 7.9% (63 mmol/mol) (SD $\pm$ 1.8%) respectively. When compared by sex, no statistically significant differences in the variables of age, body mass index (BMI), BSA, HbA1c or time of diagnosis were found.

Descriptive analysis of AHSCT and adverse effects

All patients completed the procedure on a 100% outpatient basis without severe complications. Fifteen patients required one apheresis procedure and only one subject underwent two apheresis collections in order to obtain the target number of CD34+ cells. In all cases, collection was carried out using a central venous catheter, obtaining equal to or greater than 2.0 $\times$ 10^6 CD34+ cells/kg with a mean of 11.5 $\times$ 10^6 CD34+ (SD $\pm$ 8.2). A statistically significant difference was found between sexes in the number of CD34+ cells collected. A mean of 15.8 $\times$ 10^6/kg was obtained in males, vs 6.5 $\times$ 10^6/kg in the female subgroup (P = .026). Grafting was documented at a median of 14 days (11–20 days) as well as changes in lymphocyte counts (Table 2 and Supplemental Table 6). Only one patient received extended G-CSF stimulation (7 days) and required a second collection of CD34+, giving a total count of 8.3 $\times$ 10^6/kg of body weight. The adverse effects in our study protocol were evaluated using the Common Terminology Criteria for Adverse Events v4.0, and were mainly nausea, vomiting, fever and alopecia. For nausea or vomiting, ondansetron (5 mg/m^2) was prescribed, and was an effective therapy in all cases. Four patients developed fever during the period of neutropenia, and received amoxicillin 875 mg/bid PO for 7 days. None of the patients who developed febrile neutropenia was hospitalized and the duration of the febrile illness did not exceed 48 hours in any case. Patient number 8 had an episode of grade 1 hemorrhagic cystitis (microscopic hematuria with urinary symptoms), which was managed on an outpatient basis with intense hydration in the transplant clinic. This was resolved in less than 36 hours. Platelet or red blood cell (RBC) transfusion was not required in any patient. No serious adverse effects were observed. During transplantation, neither ketoacidosis, nor symptomatic or asymptomatic hypoglycemia developed. No changes to the insulin management previously set were made.

Table 1. Basal characteristics

Group	Patient	Gender	Age (y)	Weight (kg)	Height (m)	BMI (kg/m^2)	DID (U)	Insulin (U/kg)	IA2 U/mL	Anti-GAD U/ml	Lengh of T1D	DKA	AUC C-P	HbA1c (%)
Insulin independence	1	M	17	63	1.68	22	35	0.5	<0.8	0.84	52	no	152.25	7.2
	2	M	13	56	1.59	22	23	0.4	1.4	1.0	24	yes	336.45	9.3
	3	F	11	45	1.56	18	14	0.3	7.5	2.0	36	no	188.10	8.5
	4	M	9	41	1.25	26	26	0.6	<0.8	1.0	70	yes	1003.50	8.1
	5	M	8	25	1.27	16	8	0.3	9.4	0.61	70	no	141.90	10
Mean			11.6	46	1.47	20.8	21.2	0.4	6.1	1.09	50.4		364.44	8.62
Partial insulin independence	6*	F	13	49	1.54	21	12	0.2	31.4	30	89	no	417.00	6.6
	7**	F	8	35	1.33	19	17	0.5	3.4	1.9	73	no	675.30	7.8
	8	M	15	66	1.73	22	18	0.3	>50	30	92	no	185.40	7.2
	9	F	13	43	1.60	17	13	0.3	11.8	13.3	89	no	209.10	6.5
	10	M	12	46	1.52	20	17	0.4	7.3	0.59	44	no	246.15	9.8
	11	M	12	40	1.58	16	13	0.3	5.5	30	2	no	241.05	9.2
	12	F	11	48	1.58	19	24	0.5	NA	1.2	85	no	313.80	7.8
	13	M	10	40	1.40	21	10	0.2	7.7	2.9	88	no	127.20	8.6
Mean			11.6	46	1.51	19.9	17.7	0.3	9.4	13.74	70		301.88	7.94
No response	14	M	15	50	1.62	19	28	0.5	37.6	4.7	91	no	247.80	4.1
	15	F	14	34	1.45	16	26	0.8	20.1	0.73	73	yes	228.60	5.1
	16	F	11	41	1.46	19	18	0.4	4.7	30	90	no	210.90	11.3
Mean			11.43	43	1.43	18.6	18.01	0.4	11.9	11.81	85		229.1	6.83
All groups mean			12	45	1.51	19.5	18.8	0.40	12.3	9.42	51		307.78	7.94

BMI, Body mass index

DID, Daily insulin dose (units)

SD, Standard deviation

NA, Not available

T1D, Type 1 diabetes diagnosis

DKA, Diabetic ketoacidosis

AUC-CP. Area under the curve-C-peptide

*Complete insulin independence at 11 months post-transplant

**Complete insulin independence at 12 months post-transplant.

Table 2. Transplantation characteristics

Group	Patient	Apheresis	CD34 × 10^6/kg	CD45 × $10^5/\mu l$	Days	Fever
Insulin independence	1	2	8.3	161 236	+14	No
	2	1	4.5	221 392	+11	No
	3	1	7.4	415 590	+14	No
	4	1	19.3	353 395	+14	No
	5	1	29.1	716 527	+20	No
Partial insulin independence	6*	1	2.7	100 000	+11	No
	7**	1	3.1	133 305	+14	No
	8	1	18	370 464	+16	No
	9	1	7.6	617 809	+12	Yes
	10	1	19.9	347 924	+16	No
	11	1	15.1	308 193	+14	Yes
	12	1	10.9	554 839	+14	No
	13	1	23.3	408 895	+14	Yes
No response	14	1	1.9	130 431	+14	Yes
	15	1	3.78	129 134	+13	No
	16	1	9.79	419 184	+15	No

*Complete insulin independence at 11months post-transplant

**Complete insulin independence al 12 months post-transplant.

Metabolic markers post-transplant

The median follow-up was 34 months (range 21–56 months). Changes in metabolic markers are shown in Table 3. The AUC of C-peptide in complete response group showed an increase at 6 months, but did not reach statistical significance (Figure 1). Anti-GAD titers were reduced overall by 92% compared to baseline and this was statistically significant ($P = .014$). In the group of insulin independence HbA1c values were modified downward from a mean of 8.6 to 6.38%. This corresponds to a reduction of 26%. In the group of partial responders, HbA1c values were modified downward from a mean of 7.9 to 7.0%.

Table 3. Metabolic markers at baseline and 6 months post-transplant.

Group	Patient	AUC C-P (ng/mL)		AUC Insulin		AUC Glucose		HbA1c(%/mmol/mol)		Anti-GAD(U/ml)	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Insulin independence	1	152.25	314.10	888.15	2002.80	10 935.00	13 590.00	7.20	6.00	0.84	0.62
	2	336.45	603.60	4075.50	3873.00	17 910.00	9855.00	9.30	6.00	1.00	0.52
	3	188.10	313.50	1309.20	1651.50	20 895.00	44 775.00	8.50	6.40	2.00	0.02
	4	1003.50	790.05	7671.00	8077.50	14 775.00	12 150.00	8.10	6.30	1.00	0.65
	5	141.90	141.00	1206.00	1353.30	30 960.00	25 470.00	10.00	7.20	0.61	0.56
Mean		**364.44**	**432.45**	**3029.97**	**3391.62**	**19 095**	**21 168.00**	**8.62**	**6.38**	**1.09**	**0.47**
Partial insulin independence	6	417.00	285.00	3098.25	1951.50	22 755.00	13 680.00	6.60	6.10	30.00	0.56
	7	675.30	542.85	4259.40	4886.40	18 165.00	24 510.00	7.80	5.10	1.9	1.66
	8	185.40	415.95	17 518.80	4663.50	12 345.00	15 285.00	7.20	5.00	30.00	0.66
	9	209.10	267.15	2470.50	1245.00	30 660.00	32 670.00	6.50	8.10	13.30	0.84
	10	246.15	328.50	2032.50	2984.25	23 070.00	16 830.00	9.80	5.90	0.59	0.60
	11	241.05	227.55	1877.85	1860.00	33 315.00	23 700.00	9.20	7.60	30.00	0.66
	12	313.80	225.90	1660.50	1772.40	33 120.00	51 540.00	7.80	9.90	1.20	0.89
	13	127.20	119.40	1575.90	656.85	28 905.00	36 615.00	8.60	8.30	2.9	0.58
Mean		**301.88**	**301.54**	**4311.71**	**2502.49**	**25 291.88**	**26 853.75**	**7.94**	**7.0**	**13.74**	**0.81**
No response	14	247.80	90.60	2376.90	1150.50	25 185.00	43 860.00	4.10	7.90	4.70	0.68
	15	228.60	287.25	1791.00	2469.75	16 170.00	22 230.00	5.10	5.40	0.73	0.76
	16	210.90	186.90	2923.05	1519.80	29 535.00	35 205.00	11.30	9.70	30.00	1.74
Mean		**229.1**	**188.25**	**2363.65**	**1713.35**	**23 630**	**33 765**	**6.83**	**7.67**	**11.81**	**1.06**
All groups mean		**307.78**	**321.21**	**3545.91**	**2632.38**	**23 043.75**	**26 372.81**	**7.94**	**6.93**	**9.42**	**0.75**
p-value		**0.705**		**0.284**		**0.215**		**0.072**		**0.014**	

This corresponds to a reduction of 11.4%. HbA1c levels in all groups showed a total mean decrease of 0.87% in the 3 months of study, 1% in month 6 and 1.6% in the last follow-up. At the last evaluation all patients with complete insulin independence showed a HbA1c below 7 (5.6 to 6.2%) (38 to 44 mmol/mol); on the other hand, three patients showed an increase in A1c levels above 7, all in the group of nonresponders (NR) (Figure 2). The presence of low titers of IA2 was associated with a better response, analyzing the three groups, the median in the group of complete response was the lowest (6.1 U/ml) (Table 1). Overall, the daily insulin dose requirements decreased from 0.41 U/kg to 0.32 U/kg by the third month ($P = .46$). Seven patients (44%) achieved insulin independence, five of them were free of insulin by the third month, one patient achieving total insulin independence within the first week after transplantation. Two patients with a partial insulin independence response at 6 months after the procedure showed complete insulin independence 11 months and one year after the transplant. Three patients (19%) were NR, and the remaining six (37%) showed partial insulin independence. Six months after the transplant (Supplemental Table 4), the group of complete responders was free of insulin. In the group of partial responders the daily insulin dose was reduced by 46.0% (30.0 to −88.2) and in NR, the insulin dose was increased by 67.0% (+19.0 to +150). In a univariate analysis of the three groups, there was no association between the number of CD45+ or CD34+ cells infused and the degree of response. Weight varied by between 0 and 1 kg during this trial. In the last

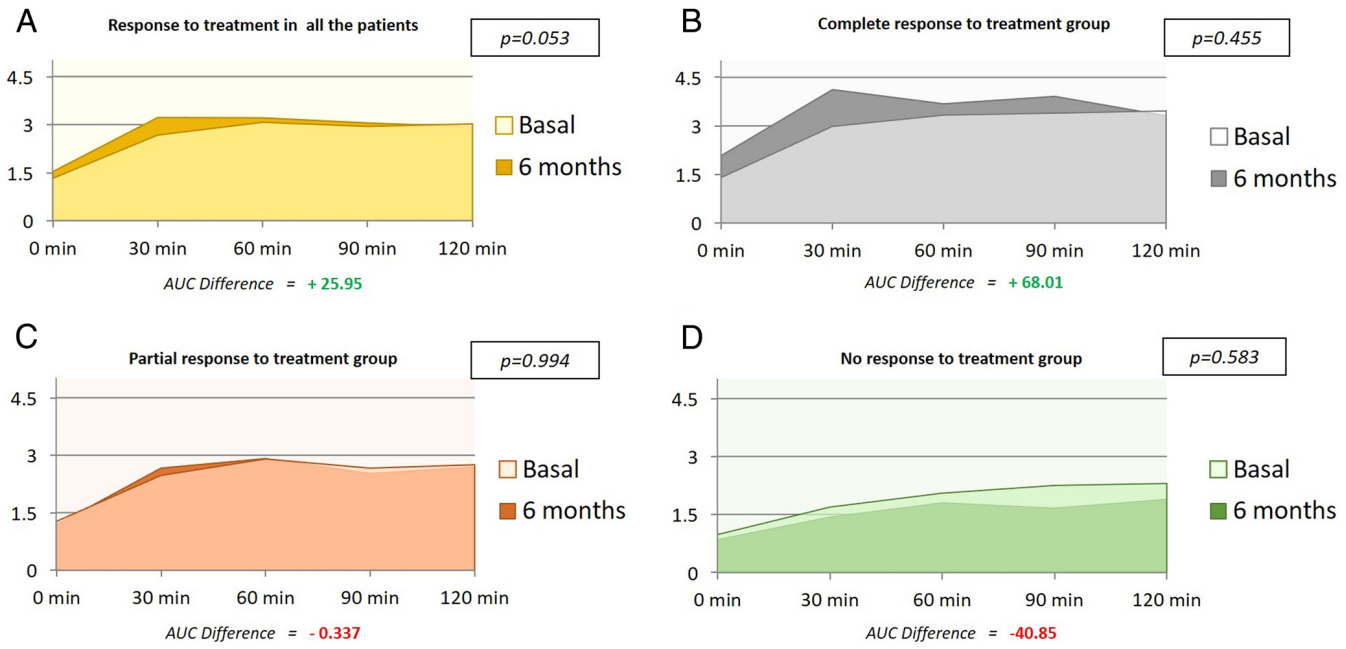

Figure 1. Patients AUC-CP basal and 6 months post-transplant in all groups.

evaluation (21–56 months) 7 patients with complete response remained without insulin and with HbA1c 6% or below (Figure 2); in the partial response group, the HbA1c was between 5.8 and 6.2%. In the control group we found a progressive increase in insulin requirement, similar to that found in the three patients in the nonresponse group, during the monitoring period no hypoglycemic events or ketoacidosis ocurred (Supplemental Table 5).

Discussion

Immune intervention in order to achieve cell preservation has fueled increasing hopes and enthusiasm as an alternative to conventional insulin replacement (29, 30). It has been shown in previous studies with median follow-ups of 18.8 to 30 months that AHSCT is a potential therapeutic strategy for early type 1 diabetes (21, 22, 25). The rationale for its use is the immune ablation obtained by this procedure, and it is based on the attractive possibility of reshaping the immune response by the chemotherapy administered during conditioning. The first successful AHSCT trial involved a conditioning regimen based on cyclophosphamide and thymoglobulin (20). This study showed that C-peptide synthesis, which mirrors insulin production in a 1:1 relationship, increased considerably, and independence of insulin injection was achieved by most patients (21). Investigators from Poland and China used a similar conditioning regimen that included cyclophosphamide 50 mg/kg/d for 4 days and ATG; interestingly the Polish group added plasmapheresis in all cases (25). It is reasonable to expect that this kind of intervention should be applied in a hospital with state-of-the-art facilities and equipped with a transplant unit. The cost of this procedure, accordingly, must be high. Complications occurred as expected, including one death (25). ATG was not considered in our simplified conditioning regimen because this drug has an elevated cost, infusion related toxicity, and requires inpatient management. Our regimen included fludarabine and low-dose cyclophosphamide, a less toxic scheme that has previously been shown to be effective in other autoimmune diseases such as aplastic anemia (31). In this study, fludarabine successfully replaced ATG. This purine nucleoside analog is active against lymphocytes by inhibiting DNA synthesis and inducing apoptosis, and it is interesting to note that CD8+ and CD4+ T cells are more affected than B cells (32). Furthermore, this drug is therapeutically efficacious because it induces lymphocytopenia and has mild nonhematologic toxicity (Supplemental Table 6) (33). It is therefore an attractive option for modifying the immune system. It is important to note that, unlike previous studies, our treatment regimen was performed fully in an outpatient setting. This, in addition to reducing costs, limits the risk of nosocomial infections. Our group has experience in outpatient transplant, using a simplified method, supported by a 7-day-a-week clinic, where medications and transfusions can be rapidly and efficiently provided if required (25). Reduced intensity conditioning regimens in AHSCT are associated with less toxicity but the risk of complications such as neutropenic fever, need for trans-

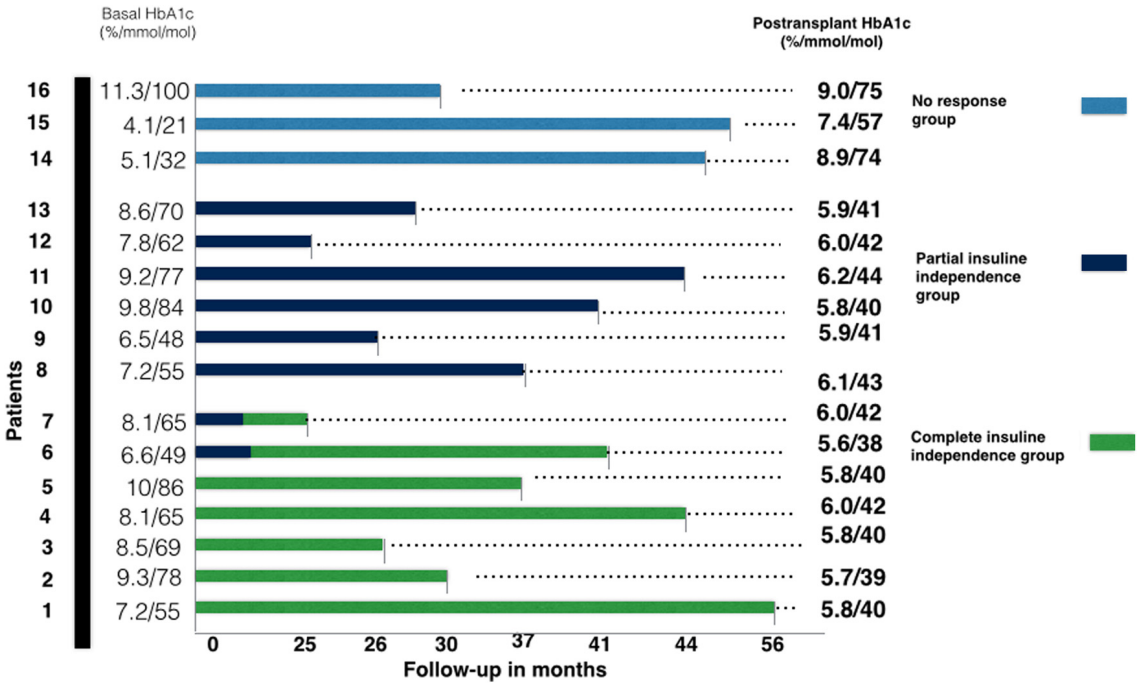

Figure 2. Changes in HbA1c per patient between basal and last follow-up determination in all groups.

fusions or hemorrhagic cystitis associated to cyclophosphamide are of concern during the early post-transplant period. Long term side effects such as damage to the reproductive system are unlikely, but the risk still exists (34 and 35). We consider that these procedures should be performed only in centers with experience in transplantation and as part of a research protocol. However, our patients were free of serious complications. On the other hand, we consider that AHSCT is a low-risk treatment in the long term. It is important to note that several trials suggest that gonadal damage is more likely to occur if a high dose of alkylating agents are administered in sexually mature patients (35). Our patients were treated at prepuberal and puberal age. In the setting of secondary malignancies, the duration of therapy is an important risk factor (36). In our cohort, patients received only four doses of cyclophosphamide. In the present study, 81% of patients increased their production and release of insulin, since all of them decreased their insulin requirements, and 44% achieved insulin independence. More importantly, this response was sustained. Although our results are not as good as those reported by Voltarelli et al (20) during the period of study, our patients did not require the expected full-blown dose of insulin for treatment of type 1 diabetes according to their age, possibly because they were recruited very early in their honeymoon phase. Furthermore, 7 patients obtained a long term complete and sustained response (median 34 months vs. 18.8 months) (20). We found that immunoablation may at least temporarily inhibit the mechanism that destroys β cells in some patients; however, as has been described in other autoimmune diseases, such as lupus or rheumatoid arthritis (RA), there is a risk of relapse. In the present study, three age groups were analyzed those younger than 12 years, 12 to 16 years and 17 years or more in order to consider the onset of adolescence and increased levels of sex hormones as potential risk factors for no response to treatment. Interestingly, four of the five patients with complete insulin independence by the third month post-transplant were either younger than 12 years or older than 17 years. In univariate analysis, there was no association between the number of CD34+cells infused, the degree of response obtained and a history of ketoacidosis prior to treatment. Therefore we can speculate that age may have some influence on the degree of response obtained. Patients with complete or partial response showed a nonsignificant increase in the level of C-peptide by the third month after transplant, but in all cases there was an improvement in the HbA1c level, with results below 7. A weakness of this study is not having a group of patients for comparison, however we included a historical control group, and is noteworthy that all of these patients showed, as expected, progressive increase in the insulin requirement. In a previous study (23), it was suggested that response to AHSCT, and therefore a beneficial effect on glycometabolic control, depended on the number of CD34+ cells injected; nevertheless, we found no statistical difference according to the amount of CD34+ and CD45+ cells infused; therefore, the need for more CD34+ cells to obtain a better response does not appear to be an absolute condition. In conclusion, our regimen, which includes fludarabine plus low-dose cyclophosphamide, led to an immunosuppressed status allowing changes in the immune system, and seemed to have less risk and fewer adverse effects than regimens involving high doses of cyclophosphamide plus ATG (23), it is also considerably less expensive. Fludarabine-based AHSCT in type 1 diabetes is a safe procedure and it is important to note that we used 4-fold less cyclophosphamide than the dose reported by Voltarelli et al (20); however, it was similar to that used in other "reduced intensity" conditioning regimens, where a total of 40–50 mg/kg is given (2800–3500 mg). The transplant was fully accomplished on an outpatient basis, thereby reducing costs, limiting exposure to nosocomial infections and avoiding the inconvenience of hospitalization. This method should be further studied in a larger cohort including an appropriate contemporary control group as it appears to be capable of changing the natural history of type 1 diabetes mellitus.

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