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Research Article

Intrabone Transplant of Cord Blood Stem Cells Establishes a Local Engraftment Store: A Functional PET/FDG Study

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Background. Despite advancements in comprehension of molecular mechanisms governing bone marrow (BM) homing of hematopoietic stem cells, cord blood transplant (CBT) suffers from a slow rate of hematopoietic recovery. Intrabone (IB) injection has been proposed as a method able to improve speed of BM engraftment with respect to conventional IV protocols. However, the mechanisms underlying this benefit are largely unknown. *Aim*. To verify whether IB-CBT determines a local engraftment able to predict the reconstitution of recipient hematopoiesis. *Design and Methods*. Twenty-one patients with hematologic malignancies received IB injection into both iliac crests of $3.2 \pm 0.68 * 107$/kg cord blood cells. One month following IB-CBT, PET-CT imaging was performed. Maximal standardized uptake values (SUVs) were assessed in BM of both iliac crests and in all lumbar vertebrae. *Results*. Maximal SUV within iliac crests was higher than in lumbar vertebrae (4.1 ± 1.7 versus 3.2 ± 0.7, resp., $P = 0.01$). However, metabolic activity in these two different BM districts was significantly correlated ($r = 0.7$, $P < 0.001$). Moreover, FDG uptake values within the injection site closely predicted platelet recovery 100 days after IB-CBT ($r = 0.72$, $P < 0.01$). *Conclusions*. The metabolic activity of injected BM predicts the subsequent rate of hematopoietic recovery after IB-CBT, suggesting a pivotal role of the local engraftment in the reconstitution of recipient hematopoiesis.

1. Introduction

Cord blood is currently used as a source of hematopoietic stem cells (HSCs) to treat a variety of malignant and nonmalignant hematologic disorders [1–5]. Its utilization has expanded beyond the pediatric field thanks to several favorable factors, including ease of collection, prompt availability, absence of risk to donors, and reduced risk of adverse effects such as graft versus host disease (GVHD) [6–8]. In adult patients, the therapeutic potential of this procedure is still limited by the low number of nucleated cells contained in each unit and by the consequent risk of delayed or inadequate recovery of neutrophils (PMN) and platelets (PLT) [9–14]. Direct cord blood cells intrabone transplantation (IB-CBT) has been proposed as a tool to improve the HSCs engraftment and thus their therapeutic effectiveness [9–14]. However, the mechanisms underlying this potential benefit have not been fully defined so far. Local HSCs engraftment might be assumed after IB-CBT, yet a recent experimental study documented that, in rats, approximately 5% of HSCs remain within the injected bone site after administration [15]. This number might indeed appear

Table 1: Main patients' characteristics.

Age	Gender	Disease	Disease status	HLA match	Overall number of infused donor cells ($\times 10^7$/kg)	Number of donor CD34$^+$ cells ($\times 10^5$/kg)	Time to PMN recovery ($\geq 0.5 \times 10^9$/L), days	Time to PLT recovery ($\geq 20 \times 10^9$/L), days	PLT count at day 100 ($\times 10^9$/kg)
66	M	nHL	Refractory	4/6	2.13	0.68	32	48	130
36	F	AML	2 CR	4/6	4.15	2.32	22	30	28
25	M	CML	Refractory	4/6	2.29	0.41	21	23	165
44	M	AML	2 CR	4/6	4.07	1.26	27	38	101
49	F	AML	1 CR (HR)	4/6	3.40	2.20	23	33	122
48	M	AML/HD	Secondary after HD	4/6	2.85	2.70	27	45	†
47	M	AML	1 CR (HR)	4/6	2.00	1.69	18	38	111
42	M	ALL	Refractory	4/6	4.40	0.86	27	13	†
34	M	HD	Refractory	4/6	3.49	1.96	24	42	124
37	M	ALL	Refractory	4/6	3.12	1.04	14	34	175
22	F	CML	Disease relapse	4/6	5.20	3.38	14	18	/
43	F	HD	Refractory	4/6	3.28	0.80	23	32	148
33	M	ALL	Refractory	4/6	3.45	1.14	23	26	77
49	F	AML	1 CR (HR)	4/6	3.87	2.67	22	35	155
50	M	AML	Refractory	4/6	2.74	0.53	29	44	100
58	M	nHL	Refractory	4/6	2.58	1.58	24	38	127
46	F	CML	Disease relapse	4/6	1.97	1.60	24	39	/
38	M	AML	2 CR	4/6	2.19	1.00	19	37	212
36	M	AML	Refractory	4/6	2.46	1.00	19	29	†
56	M	CML	Refractory	4/6	2.90	1.67	19	34	118
58	M	PMF	Refractory	4/6	3.36	1.71	24	36	51

ALL: acute lymphatic leukemia; AML: acute myeloid leukemia; CML: chronic myeloid leukemia; HD: Hodgkin's disease; nHL: non-Hodgkin's lymphoma; PMF: primary myelofibrosis; 1 CR: first complete remission; 2 CR: second complete remission; HR: high risk; PMN: polymorphonucleated cells; PLT: platelets; †Patient died.

extremely low. It, however, favorably compares with the 10% seeding efficiency of conventional protocols implying intravenous administration [16, 17]. In fact, should the same homing features characterize transplanted HSCs present in the circulating blood, the extra local engraftment produced by IB-CBT might play a relevant role in accelerating the reconstitution of recipient hematopoiesis.

In order to test this hypothesis, we verified whether the metabolic activity in the site of injection predicts the subsequent reconstitution of recipient hematopoiesis in adult patients treated with IB-CBT. To this purpose, we exploited the high sensitivity of ^{18}F-fluorodeoxiglucose (FDG) PET-CT imaging. This method has been proved to accurately estimate bone marrow (BM) mitotic activity as well as its hematopoietic response to chemotherapy in a large number of conditions. In this setting, this technique provided us with the advantage to simultaneously evaluate BM metabolism, as an index of proliferating activity, within the injected bone as well as in remote sites. The subsequent monitoring of peripheral blood cell reconstitution confirmed the main role of injected sites in the early phases of hematopoietic recovery.

2. Design and Methods

2.1. Study Group and Transplant Preparation. The study group included 21 subjects (15 males, age 43 ± 11 years, range 22–66) who were submitted to IB-CBT according to the indications reported in Table 1. In all patients, cord blood was used as a source of HSCs due to the absence of human-leucocyte-antigen-(HLA-) matched donors in a clinically useful time frame. Patients' characteristics are shown in Table 1. Treatment plan was reviewed and approved by the local ethical committee and written informed consent was obtained from all patients before any treatment.

As requested by our standard operative treatments, conditioning regimen was accomplished by an integrated approach. All patients received fractionated 10–12 Gy total body irradiation on days −6, −5, and −4 before transplantation (subdivided into one fraction per day or fractions of 2 Gy twice a day). Drug treatment included the administration of thiotepa (8 mg/kg) on day −8, treosulfan (10–12 g/m^2 daily) on days −7, −6, and −5, fludarabine (40 mg/m^2 per day) from day −7 to day −3, and cyclophosphamide (60 mg/kg per day) on days −2 and −1.

All patients received a combination of cyclosporine, mycophenolate mofetil, and antithymocyte globulin as GVHD prophylaxis. Intravenous cyclosporin was started on day −7 at a daily dose of 1 mg/kg and, in the absence of GVHD, was tapered until discontinuation from day +90 to day +180 after transplantation. The dose of cyclosporin was adjusted in order to maintain serum through concentration within the 150–300 mg/L range. Mycophenolate mofetil was given

Table 2: Main clinical characteristics and SUV values of control subjects.

	Age	Gender	Disease	Iliac crest SUV	Vertebrae SUV
	48	M	NHL	1.82	3.98
	38	M	NHL	1.96	4.91
	70	M	NHL	1.35	3.23
	35	F	HD	1.92	2.90
	65	M	HD	2.38	3.85
	63	M	NHL	2.09	3.39
	59	F	NHL	0.65	3.61
	35	M	HD	1.96	6.13
	39	M	HD	1.47	4.97
	51	M	NHL	3.07	5.89
	38	M	NHL	1.04	2.71
	57	F	NHL	1.81	4.68
	40	M	NHL	2.24	8.35
	46	M	NHL	2.00	4.34
	55	M	NHL	2.18	3.78
	39	F	HD	0.62	2.15
	31	M	HD	1.85	3.12
	30	M	HD	1.83	3.05
Mean ± SD	47 ± 12			1.79 ± 0.60	4.17 ± 1.49

at a dose of 15 mg/kg orally twice a day in the first 28 days after IB-CBT. Antithymocyte globulin was given at a dose of 3 mg/kg per day on days −3 and −2. No patient received steroids. All patients received granulocyte colony stimulating factor.

2.2. Control Group. To verify the dependence of FDG uptake upon the history of bone trauma within the IB-CBT site, we evaluated a group of 18 patients (14 males, mean age 47 ± 12 years, range 19–65) as control. They were submitted to PET-CT scanning 25–40 days after BM aspiration biopsy from the iliac crests. These evaluations were performed in the course of followup, two months after the last chemotherapy application for lymphoma. Patients previously exposed to radiotherapy were excluded from this analysis. Demographic data and imaging findings in this population are reported in Table 2.

2.3. Cord-Blood-Unit Processing and Intrabone Transplant. Cord-blood units were obtained from several cord-blood banks. Before IB-CBT, surface expression of CD34 antigen was determined after thawing with a FACSAria flow cytometer using a single platform protocol (ISHAGE). Total number of nucleated cells was in all cases greater than $1 * 10^7$ per kilogram of recipient body weight, as determined before freezing. Cord-blood units were thawed in a 37°C water bath, according to the procedure described by Rubinstein et al. [18]. To remove dimethyl-sulphoxide, cells were washed with saline plus dextran and human albumin. The same solution was used to prepare a 20 mL cell suspension that was divided into four 5-mL syringes for administration.

Before the procedure, patients were sedated with intravenous propofol and positioned in the flank posture. A standard needle for BM aspiration (14 gauge) was inserted into a superior-posterior iliac crest; an aspiration of about 1 mL was then executed in order to ascertain that the needle was securely inserted into BM cavity. Subsequently, each cord-blood-cell suspension was gently infused into the intrabone space. This procedure was then repeated for all the remaining aliquots at a distance of about 3–5 cm from each other. In all cases, an uneven distribution of injections was used, randomly selecting the side subjected to three or one administration.

2.4. Followup. Peripheral blood cell counts was estimated daily for the first month after IB-CBT and twice weekly thereafter. For the purposes of the present study, the last time point was set at 100 days. PLT counts, obtained using standard methods, were thus considered as marker of hematopoiesis reconstitution. PMN counts were also monitored to complete the description of blood cells populations. Growth of colony forming cells (CFC) was assessed by use of a complete semisolid medium (Methocult, Stem Cell Technologies Inc., Vancouver, BC, Canada) under standard conditions at day +30 and +100 after BM transplant.

2.5. PET-CT Acquisition. PET-CT imaging of FDG distribution was performed 30 days following BM transplantation. After 6 hours fasting, serum glucose level was measured before intravenous injection of 4.8–5.2 MBq of 18F-FDG per kilogram of body weight. All these procedures were performed in a quiet room with the patient recumbent in supine position and instructed not to move. FDG-PET imaging, from vertex to toes, started 60 to 90 minutes after tracer administration and was performed using an integrated PET/CT scanner (Hirez; Siemens Medical Solutions, Knoxville TN, USA).

2.6. Image Analysis. PET raw data were reconstructed by means of ordered subset expectation maximization (OSEM) and attenuation correction was performed by means of CT. The entire CT dataset was coregistered with the 3-dimensional PET data using an integrated software interface (Syngo Image Fusion; Siemens Erlangen, Germany) to combine anatomical and functional images in all body districts. FDG uptake was semiquantitatively evaluated in the injection sites as well as in remote BM. To this purpose, volumetric regions of interest (VROIs) were manually drawn on the anatomic CT images in order to identify the trabecular space within each iliac crest as well as in the soma of all five lumbar vertebrae (Figure 1). Maximal standardized FDG uptake values (SUVs) were measured in these three districts to estimate the metabolic activity of both injection sites and remote BM. The extension of the metabolic effect produced by IB-CBT in both iliac crests was also evaluated. To this purpose, we applied a criterion previously described byKidd and Grigsby to measure the total mass of metabolically active solid cancer [19]. This method, implemented in Syngo software, provides an estimation of the total volume included in

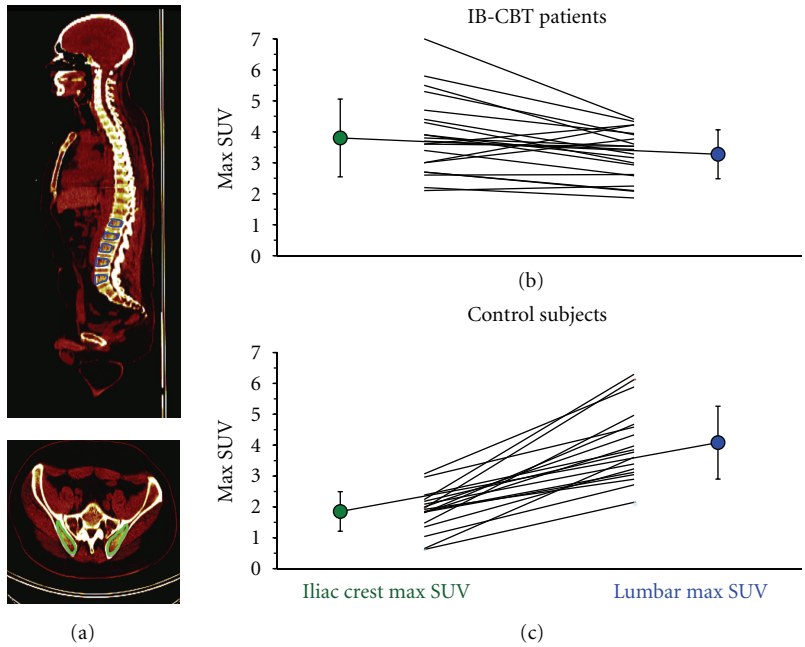

FIGURE 1: (a) Bone marrow (BM) segmentation of both iliac crests (bottom) and five lumbar vertebrae (top), using PET-CT images. Analyzed volumes are shown in green for the former and blue for the latter. (b) Metabolic activity trend patient by patient in the injected sites compared with remote BM; maximal SUVs in the iliac crests were slightly but significantly higher than in the lumbar spine. On the contrary, the same analysis (c) showed the opposite results in controls.

the iliac crest VROI presenting FDG uptake values scoring at least 40% of maximum SUV. Once defined in each iliac crest, this volume was multiplied for the corresponding average SUV providing an integrated index of both extension and metabolic activity of engrafted BM (the so-called "metabolic volumetric product").

2.7. Statistical Analysis. All data are reported as means ±SD. Unpaired or paired t-test was used, as appropriate, to compare the same variables at the three different time points. Linear regression analysis was performed using the least squares method. P value <0.05 was considered significant.

3. Results

3.1. Hematopoietic Engraftment. Cord blood unit was mismatched with the recipient for 2/6HLA antigen and the disparity was in class I at the antigenic level. Infused cell dose was 3.2 ± 0.68 (range 1.9–5.1) $* 10^7$ cells/kg. Number of $CD34^+$ cells infused was $1.5 \pm 1.0 * 10^5$/kg. No immediate side effects, such as pain, hemorrhage, or local infections, were recorded.

Three out of 21 (14%) patients died within 50 days from the procedure due to multiorgan failure. In two further patients, PLT counts at day 100 were not considered because of evidence of disease relapse. The remaining patients achieved complete hematological recovery as documented by the monitoring of peripheral blood cells counts and remained in remission thereafter. In fact, PMN concentration was $0.019 \pm 0.009 * 10^9$/L at the time of BM transplantation (day 0), it increased to $3.9 \pm 2 * 10^9$/L ($P <$ 0.0001 versus baseline) at day 50, and remained relatively stable thereafter until day 100 ($4.6 \pm 1.7 * 10^9$/L, P = ns versus day 50). Despite the needed transfusion before IB-CBT, PLT counts showed a different pattern, progressively rising from day 0 ($3.1 \pm 1.1 * 10^9$/L) to $82 \pm 50 * 10^9$/L at day 50 ($P < 0.01$ versus day 0), up to $122 \pm 46 * 10^9$/L at day 100 ($P < 0.01$ versus both previous temporal milestones).

PMN and PLT recovery was considered established when these elements reached for five consecutive days and in the absence of transfusions a steady and consistent concentration greater than $0.5 * 10^9$/L and $20 * 10^9$/L, respectively. As expected, this event occurred earlier for PMN than for PLT (22.6 ± 4.5 versus 34 ± 8.6 days, $P < 0.001$).

Among surviving patients, acute GVHD incidence and severity were graded at day 100 as 0 ($n = 6$), 1 ($n = 8$), 2 ($n = 3$), or 3 ($n = 1$) according to conventional criteria [20].

3.2. Extension and Metabolic Activity of Bone Marrow within the Injected Sites. No patient showed areas of abnormal FDG uptake diagnostic for neoplastic localization within the skeleton or in the rest of the body. However, FDG uptake in the iliac crests was clearly visible and it was most often higher than in the rest of BM (Figure 2). In particular, with respect to the soma of the five lumbar vertebrae, injected bone districts showed significantly higher maximal SUVs (4.1 ± 1.7 versus 3.2 ± 0.7, resp., $P = 0.01$; Figure 1). This pattern was a specific prerogative of patients submitted to IB-CBT. In fact, in control group, SUVs presented the opposite pattern being lower within iliac crests with respect to lumbar vertebrae (1.8 ± 0.6 versus 4.2 ± 1.5, resp., $P < 0.001$; Figure 1).

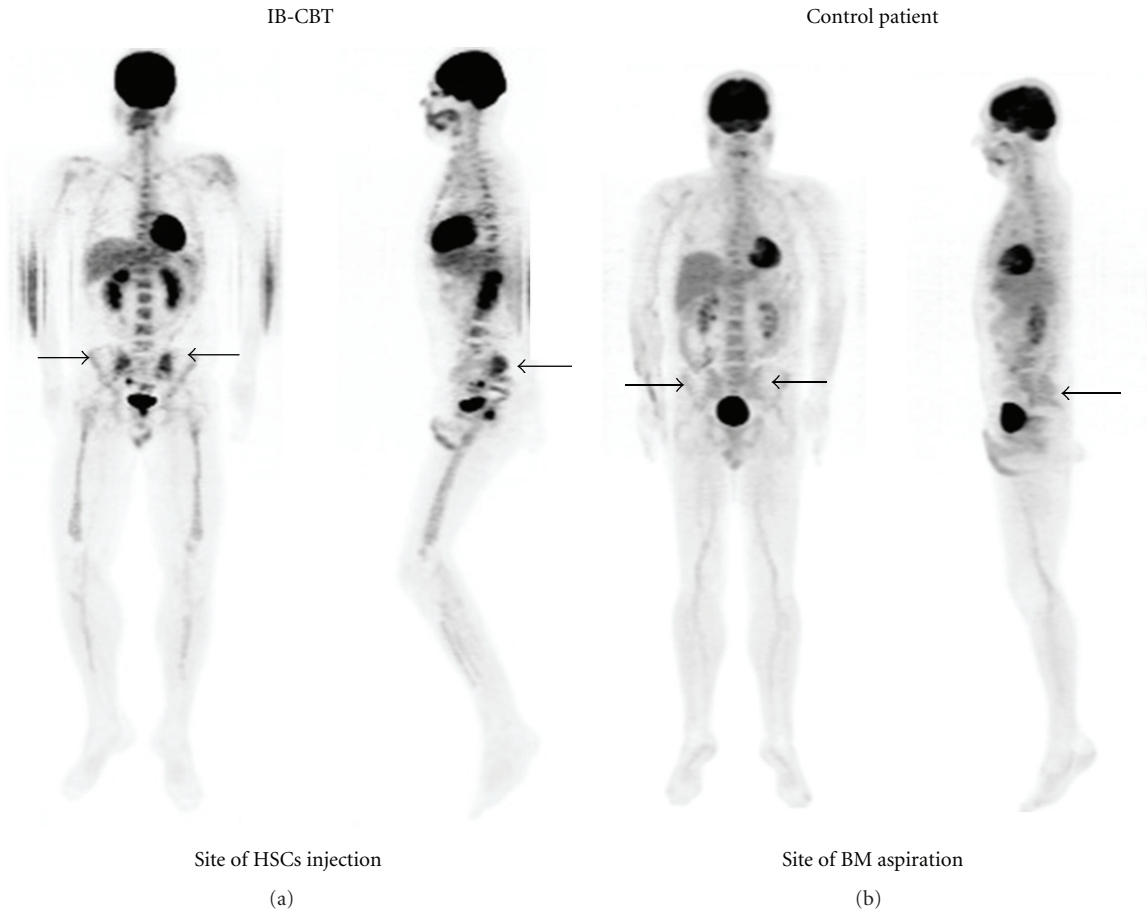

Figure 2: Whole body PET maximum intensity projections of a patient (a) and a control subject (b). Tracer retention in iliac crests is clearly visible in the patient and not in the control subject.

This finding was also confirmed by the fact that SUVs were significantly higher within the injected bone of transplanted patients than in the corresponding BM segments of controls (4.1 ± 1.7 versus 1.8 ± 0.6, resp., $P < 0.0001$). Conversely, lumbar BM metabolic activity showed the opposite, though less evident, difference being lower in IB-CBT patients than in controls (3.2 ± 0.7 versus 4.2 ± 1.5, resp., $P < 0.05$; Figure 1).

Furthermore, iliac crests submitted to three injections showed higher SUVs than those treated with only one (4.3 ± 1.1 versus 3.3 ± 0.9, resp., $P < 0.01$). This could suggest the existence of a link between the number of injected cells and local metabolic increase. This concept is further confirmed by the fact that a trend toward the correlation was observed between metabolic activity in the injected bone and number of locally injected $CD34^+$ cells ($r = 0.35$, $P = 0.12$).

3.3. BM Metabolic Activity and Hematopoietic Recovery. To verify whether BM metabolic activity was related to the speed of hematopoietic recovery, we divided the population into two groups according to the median interval between IB-CBT and recovery of cell counts. Dividing the population according to the median interval needed for PMN recovery did not show any significant different maximal SUVs, in neither iliac crest nor in remote BM between the two groups. On the contrary, when PLT renewal was considered, patients who presented faster repopulation (recovery occurring before the median day 35) displayed higher maximal SUVs than the slow-recovery group. This metabolic increase was evident within the injected site (4.43 ± 1.7 versus 2.9 ± 0.8, resp., $P < 0.05$), but it was not significant within lumbar vertebrae (3.5 ± 0.7 versus 3.1 ± 0.8, resp., $P =$ ns). Intensity and extension of metabolic activity in both injected sites predicted the reconstitution of recipient hematopoiesis at day 100. In fact, a close direct correlation was observed between maximal SUVs in the site of injection and in remote BM ($r = 0.70$, $P < 0.001$) (Figure 3). Metabolic activity in both iliac crests was unrelated to PLT counts both at time of transplant ($r = 0.32$, $P =$ ns) and at day 50 ($r = 0.26$, $P =$ ns); however, it robustly predicted PLT counts at day 100 ($r = 0.72$, $P < 0.001$; Figure 4). This correlation was remarkably less evident for remote BM. In fact, maximal SUV in lumbar vertebrae did not correlate with PLT counts neither at day 0 ($r = 0.06$, $P =$ ns) nor at day 50 ($r = 0.28$, $P =$ ns), while it approached the statistical significance threshold at day 100 ($r = 0.31$, $P = 0.06$) (Figure 5). This finding was confirmed and corroborated by data related to the volumetric extension of HSCs engraftment in the site of injection. In fact, the volume encompassing voxels with SUVs

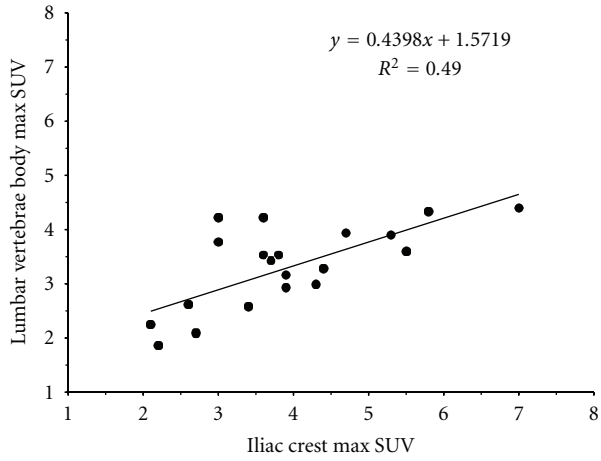

Figure 3: Correlation existing between maximal standardized FDG uptake values (SUV) within iliac crests and lumbar vertebrae. The tight correlation between injected sites and lumbar vertebrae metabolism confirmed a close parallelism between the degree of BM glucose consumption in these two different districts.

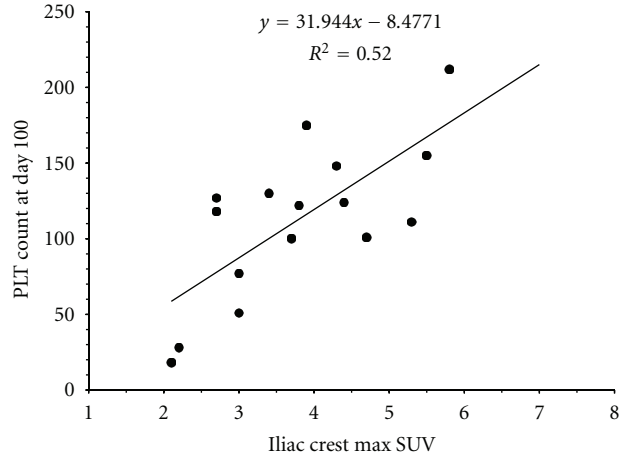

Figure 4: Correlation between follow-up platelets counts (day 100) and metabolic activity in both the iliac crests at day 30 after IB-CBT; maximal SUVs in the injected sites were significantly and directly correlated with the follow-up platelets recovery.

≥40% of the maximum was 2.5 ± 0.5 mL and correlated with late follow-up PLT counts ($r = 0.64$, $P < 0.01$). Similarly, the metabolic volumetric product in the sites of injection showed an even closer correlation with PLT counts at the end of the study ($r = 0.75$, $P < 0.01$).

4. Discussion

The present study aimed to verify whether hematopoiesis recovery after IB-CBT is at least partially explained by the local increase in BM metabolism, as an index of HSCs proliferating activity, within the injection site. Mapping BM glucose consumption throughout the whole body confirmed this hypothesis. FDG uptake was higher in iliac crests than in remote BM and it was dependent upon the number of

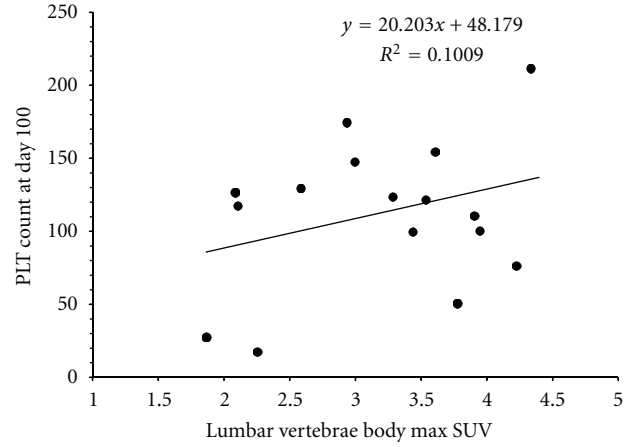

Figure 5: Lack of correlation between late follow-up platelets count (day 100) and maximal SUVs in lumbar vertebrae; the metabolic activity in the remote BM did not predict the late hematopoietic recovery.

locally administered cord blood cells. The degree of glucose consumption in the site of injection was strictly correlated with the metabolic pattern in lumbar vertebrae, suggesting a role for locally engrafted cells in determining cell density and metabolism in remote BM. PLT counts recovery at day 100 was accurately predicted by degree and extension of metabolic activity in the injected bone and only loosely by FDG uptake in remote BM. Although the mechanisms underlying this potential benefit are far beyond the scope of the present study, these data suggest that IB-CBT could induce a local "engraftment store" capable to transiently accelerate the hematopoietic recovery and thus to bridge the traditional gap that limited the clinical potential for cord blood transplantation in adult patients [13].

4.1. FDG Distribution after IB-CBT. The potential role of FDG imaging in evaluating hematopoietic function has been documented by a number of studies reporting a biphasic response of BM SUV to chemotherapy with a reduction during cytotoxic treatment followed by an increase during the proliferating reaction in the subsequent recovery phase [21–23]. In hematologic patients, the presence of residual disease might partially limit the interpretation of tracer uptake as an index of HSCs metabolism. However, several considerations suggest that this possibility should have played a relatively minor role in the present study. In fact, all patients were exposed to a complex regimen encompassing chemotherapy and total body irradiation before IB-CBT. Moreover, the focal hot spots typical of residual disease did never occur in our patients who rather displayed a homogeneous and diffuse increase in BM FDG uptake, with a relatively higher tracer retention selectively involving the injected iliac crests. Accordingly, although a small number of diseased cells might have been present in some patients, their dispersed nature should have been not recognizable due to the limited spatial resolution of PET/CT imaging.

In agreement with these considerations, the high sensitivity of whole-body FDG imaging permitted to simultaneously evaluate the metabolic activity in the injected bone and in the remote BM. The comparison with a control group of patients studied 20–40 days after iliac crest biopsy rules out the possible interference on BM metabolism caused by the bone trauma following injection. In fact, these patients not only showed lower SUVs in iliac crests with respect to subjects treated with IB-CBT, rather they also displayed an opposite tracer distribution, presenting highest uptake values in lumbar vertebrae bodies. Accordingly, both observations strongly and concordantly indicate that the enhancement in iliac crests metabolism in transplanted patients is the consequence of local HSCs administration rather than a possible metabolic consequence of previous bone trauma or residual disease.

This concept is further supported by the observation that FDG uptake in each injected bone was dependent on the corresponding number of locally administered cells. Moreover, the persistence of this "cell-dose effect" one month after IB-CBT corroborates the concept that the increased glucose consumption represents both an index of a high cell density and a marker of increased proliferating activity within the site of injection.

4.2. BM Metabolic Pattern and Hematopoietic Recovery. The volume of metabolically active HSCs within the iliac crests was estimated using a method validated for studies dealing with BM metabolism and cancer evaluation [19, 24] and appears too small to explain the restored "production" of circulating blood cells. On the other hand, FDG uptake in the injection site was directly correlated with the corresponding value in lumbar vertebrae. Moreover, metabolic pattern in iliac crests closely predicted both the day of PLT "normalization" as well as PLT concentration 100 days after transplant. These findings suggest that metabolic rate of locally engrafted cells profoundly contributes to remote BM colonization, although this process is not yet completed at one month after transplant when FDG uptake in lumbar vertebrae only loosely correlates with the subsequent hematopoietic recovery.

In line with the notion that megakaryopoiesis is a more accurate indicator for the quality of HSCs function than granulopoiesis [13, 25–27], neutrophil recovery was not clearly correlated with our imaging indexes. However, neutrophil counts display a slower recovery with respect to PLT [19, 27] and a large variability due to the interference of numerous confounding factors. Both these features hamper the analysis of a possible correlation between HSCs metabolism and granulocyte counts in a small population sample. Thus, independently from the extremely relevance of leukocyte reconstitution in determining patients survival, PLT count was more suited for the purpose of the present study.

5. Conclusion

Present data indicate that direct injection of cord blood cells into BM spaces facilitates their in situ proliferating activity. The initial benefit is probably followed by an improved migration of stem cells to remote uninjected BM sites. Altogether, these effects at least partially explain the accelerated hematopoietic recovery observed after IB-CBT [13]. The present study does not provide any insight into the clinical potential and effectiveness of IB-CBT that has been already reported in the literature [13]. Nevertheless, the robustness of our findings and their agreement with previous experimental and clinical evidence indicate that IB-CBT might represent a useful model to elucidate the biology of donor HSCs in the recipient.

Authors' Contribution

C. Marini, M. Piana, A. Bacigalupo, F. Frassoni, and G. Sambuceti contributed to the conception of the study and relative planning; C. Marini, M. Podestà, M. Massollo, S. Capitanio, F. Fiz, and S. Morbelli carried out the analysis of the data and interpretation of the data. All the authors contributed to the writing, discussion, and approval of the paper.

Conflict of Interests

The authors declare no conflict of interests.

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