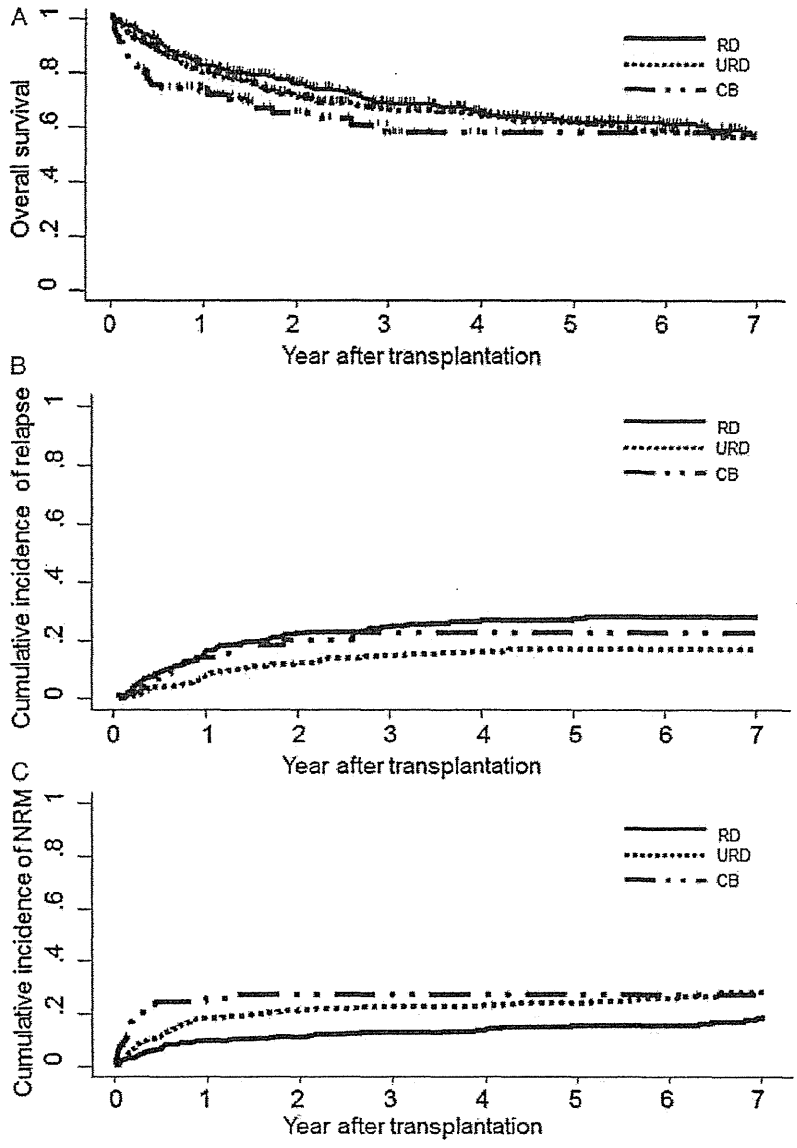

Figure 1. Transplant outcomes by a donor source for 917 Ph(−) ALL patients who underwent myeloablative allo-SCT in CR1. (A) Overall survival; (B) cumulative incidence of relapse; (C) cumulative incidence of non-relapse mortality (NRM). RD, related donor; URD, unrelated donor; CB, cord blood.

0.83–1.44), $P = 0.53$; CB: HR 1.20 (95% CI 0.76–1.89), $P = 0.43$ (versus RD)]. Among CB recipients in CR1, only age at allo-SCT (≥45 years) was a significant prognostic factor in multivariate analysis [HR 2.89 (95% CI 1.43–5.81), $P = 0.003$]. CD34-positive cell dose was not a significant prognostic factor. Subgroup analyses of CR1 patients younger than 45 years showed that OS after CB allo-SCT ($N = 65$) was significantly better than that after mismatched URD allo-SCT ($N = 47$) in CR1 (68% versus 49% at 4 years, $P = 0.04$).

relapse and NRM among patients transplanted in CR1

The cumulative incidence of relapse was significantly lower in patients who underwent URD allo-SCT than in those who underwent RD or CB allo-SCT (25% in RD, 17% in URD, and 22% in CB at 3 years; $P = 0.02$) (Figure 1B). The results of multivariate analysis showed that abnormal cytogenetics, non-TBI preparative regimens, and RD were significant risk factors for relapse (Table 2).

The cumulative incidence of NRM was significantly lower in patients who underwent RD allo-SCT than in those who underwent URD or CB allo-SCT (13% in RD, 23% in URD, and 27% in CB at 3 years; $P = 0.0001$) (Figure 1C). The results of multivariate analysis showed that ≥45 years of age at allo-SCT, JALSG intermediate or high risk, HLA partially matched or mismatched, non-TBI preparative regimens, transplantation between 1998 and 2004, <6 or ≥10 months from diagnosis to allo-SCT, and URD were significant risk factors for NRM (Table 2).

Table 2. Outcomes for adult Ph(−) ALL patients undergoing myeloablative allo-SCT in CR1: multivariate analyses ($N = 917$)

Covariates	Relative risk (95% CI)	P
Overall survival		
Favorable		
Year of allo-SCT (versus 1998–2004)		
2005–2009	0.75 (0.59–0.95)	0.02
Unfavorable		
Age at allo-SCT, year (versus 16≤, <45)		
45	1.73 (1.33–2.25)	<0.0001
JALSG risk (versus low)		
Intermediate	1.36 (1.01–1.82)	0.04
High	1.54 (1.06–2.22)	0.02
HLA (versus well matched)		
Partially matched	1.55 (1.87–2.02)	0.001
Mismatched	1.60 (1.11–2.31)	0.01
Conditioning (versus TBI regimens)		
Non-TBI regimens	2.02 (1.35–3.03)	0.001
Interval from diagnosis to allo-SCT, month (versus 6≤, <10)		
<6	1.42 (1.09–1.85)	0.009
Relapse		
Favorable		
Source (versus related)		
Unrelated BM	0.61 (0.44–0.84)	0.002
Unfavorable		
Cytogenetics (versus normal)		
Others [no hypodiploid, $t(4;11)$, $t(8;14)$, nor $t(9;22)$]	1.42 (1.05–1.93)	0.02
Conditioning (versus TBI regimens)		
Non-TBI regimens	1.90 (1.05–3.42)	0.03
Non-relapse mortality		
Favorable		
Year of allo-SCT (versus 1998–2004)		
2005–2009	0.60 (0.43–0.83)	0.002
Unfavorable		
Age at allo-SCT, year (versus 16≤, <45)		
≤45	2.46 (1.78–3.39)	<0.0001
JALSG risk (versus low)		
Intermediate	1.82 (1.16–2.86)	0.009
High	2.00 (1.36–3.40)	0.01
HLA (versus Well matched)		
Partially matched	1.81 (1.24–2.66)	0.002
Mismatched	2.13 (1.36–3.34)	0.001
Conditioning (versus TBI regimens)		
Non-TBI regimens	2.10 (1.21–3.64)	0.009
Interval from diagnosis to allo-SCT, month (versus 6≤, <10)		
<6	1.76 (1.19–2.61)	0.005
10≤	1.43 (1.00–2.06)	0.048
Source (versus related)		
Unrelated BM	1.53 (1.04–2.24)	0.03
Cord blood	1.64 (0.93–2.87)	0.09

Ph(−), ALL indicates Philadelphia chromosome-negative acute lymphoblastic leukemia; allo-SCT, allogeneic stem cell transplantation; CR, complete remission; TBI, total body irradiation; BM, bone marrow.

causes of death among patients transplanted in CR1

The frequency of relapse was significantly higher in patients who underwent RD allo-SCT ($P = 0.001$). Infection, organ failure, GVHD, and interstitial pneumonia were the major causes of NRM, and the incidence of hemorrhage was significantly higher in patients who underwent CB allo-SCT ($P = 0.009$) (Table 3).

other outcomes of allo-SCT among patients transplanted in CR1

The cumulative incidence of neutrophil recovery or platelet recovery was significantly lower in patients who underwent CB allo-SCT (neutrophil recovery: 98% in RD, 98% in URD, and 72% in CB at day 100, $P < 0.0001$; platelet recovery: 95% in RD, 91% in URD, and 81% in CB at 1 year, $P < 0.0001$). Among CB

Table 3. Causes of death for adult Ph(−) ALL patients undergoing myeloablative allo-SCT in CR1 ($N = 917$)

	Related		Unrelated BM		Cord blood		
No. of patients	130	(%)	148	(%)	33	(%)	P
Relapse	59	45	37	25	8	24	0.001
Infection	17	13	26	18	8	24	0.26
Organ failure	17	13	23	16	1	3	0.15
GVHD	11	8	19	13	2	6	0.34
Interstitial pneumonia	8	6	15	10	4	12	0.38
Hemorrhage	3	2	7	5	5	15	0.009
TMA	7	5	3	2	2	6	0.27
ARDS	1	1	5	3	0	0	0.20
Graft failure	2	2	2	1	2	6	0.18
SOS	2	2	2	1	0	0	0.78
Secondary malignancy	1	1	0	0	0	0	0.50
Other	2	2	9	6	1	3	0.14

Ph(−), ALL indicates Philadelphia chromosome-negative acute lymphoblastic leukemia; allo-SCT, allogeneic stem cell transplantation; CR, complete remission; BM, bone marrow; GVHD, graft-versus-host disease; TMA, thrombotic microangiopathy; ARDS, acute respiratory distress syndrome; SOS, sinusoidal obstruction syndrome.

recipients, time to neutrophil engraftment was associated with a CD34-positive cell number ($<1 \times 10^5$/kg: day 22 versus $\geq 1 \times 10^5$/kg: day 19, $P = 0.02$).

The cumulative incidence of grade III–IV acute GVHD was significantly higher in patients who underwent URD allo-SCT (8% in RD, 18% in URD, and 11% in CB at day 100; $P = 0.008$).

Among assessable patients who survived at least 100 days after allo-SCT, no significant difference was observed between RD, URD, and CB allo-SCTs in the incidence of chronic GVHD (34% in RD, 38% in URD, and 31% in CB at 3 years; $P = 0.52$)

allo-SCT in subsequent CR

Although it was concluded from the results of a study by MRC/ECOG that RD allo-SCT in CR1 could achieve the best result [10], there is still plenty of room to discuss allo-SCT beyond CR1 for patients who could not find a suitable donor or maintain CR1. Among 300 patients transplanted in subsequent CR, there were no significant differences in OS between RD, URD, and CB allo-SCTs (47% in RD, 39% in URD, and 48% in CB at 4 years; $P = 0.33$). The results of multivariate analysis showed that JALSG intermediate- or high-risk and cytogenetic abnormalities [hypodiploid, $t(4;11)$ or $t(8;14)$] were significant risk factors for OS (Table 4). The donor source was not a significant risk factor [URD: HR 1.28 (95% CI 0.90–1.82), $P = 0.17$; CB: HR 1.01 (95% CI 0.61–1.67), $P = 0.97$ (versus RD)].

The cumulative incidence of relapse was not statistically different among patients who underwent RD, URD, and CB allo-SCTs (31% in RD, 26% in URD, and 29% in CB at 3 years; $P = 0.48$). The results of multivariate analysis showed

Table 4. Outcomes for adult Ph(−) ALL patients undergoing myeloablative allo-SCT in subsequent CR: multivariate analyses ($N = 300$)

Covariates	Relative risk (95%CI)	P
Overall survival		
JALSG risk		
Low	1.00	
Intermediate	1.45 (1.05–2.02)	0.03
High	1.92 (1.08–3.41)	0.03
Cytogenetics		
Normal	1.00	
Hypodiploid, $t(4;11)$ or $t(8;14)$	2.48 (1.11–5.52)	0.03
Others [no $t(9;22)$]	1.24 (0.89–1.71)	0.20
Relapse		
Cytogenetics		
Normal	1.00	
Hypodiploid, $t(4;11)$ or $t(8;14)$	4.13 (1.54–11.1)	0.005
Others [no $t(9;22)$]	1.29 (0.82–2.02)	0.27
Non-relapse mortality		
Age at allo-SCT, year		
16≤, <45	1.00	
≥45	1.82 (1.08–3.07)	0.02
JALSG risk		
Low	1.00	
Intermediate	1.52 (0.94–2.47)	0.09
High	2.39 (1.12–5.10)	0.02

Ph(−) ALL indicates Philadelphia chromosome-negative acute lymphoblastic leukemia; allo-SCT, allogeneic stem cell transplantation; CR, complete remission.

that cytogenetic abnormalities [hypodiploid, $t(4;11)$, or $t(8;14)$] were significant risk factors for relapse (Table 4).

Similarly, the cumulative incidence of NRM was not statistically different among patients who underwent RD, URD, and CB allo-SCTs (21% in RD, 36% in URD, and 27% in CB at 3 years; $P = 0.46$). The results of multivariate analysis showed that ≥45 years of age at allo-SCT and JALSG intermediate or high risk were significant risk factors for NRM (Table 4).

allo-SCT in non-CR

Among 509 patients transplanted in non-CR, there were no significant differences in OS among patients who underwent RD, URD, and CB allo-SCTs (15% in RD, 21% in URD, and 18% in CB at 4 years; $P = 0.20$). The results of multivariate analysis showed that ≥45 years of age at allo-SCT, cytogenetic abnormalities, HLA partially matched or mismatched, and non-TBI preparative regimens were significant risk factors for OS (Table 5). The donor source was not a significant risk factor [URD: HR 0.99 (95% CI 0.79–1.24), $P = 0.96$; CB: HR 1.09 (95% CI 0.78–1.53), $P = 0.61$ (versus RD)].

The cumulative incidence of relapse was not statistically different among patients who underwent RD, URD, and CB allo-SCTs (59% in RD, 42% in URD, and 58% in CB at 3 years; $P = 0.35$). However, the results of multivariate analysis showed that the donor source as well as cytogenetic abnormalities [hypodiploid, $t(4;11)$, or $t(8;14)$] and non-TBI

Table 5. Outcomes for adult Ph(−) ALL patients undergoing myeloablative allo-SCT in non-CR: multivariate analyses ($N = 509$)

Covariates	Relative risk (95% CI)	P
Overall survival		
Age at allo-SCT, year		
16≤, <45	1.00	
≥45	1.65 (1.30–2.11)	<0.0001
Cytogenetics		
Normal	1.00	
Hypodiploid, $t(4;11)$ or $t(8;14)$	2.04 (1.38–3.03)	<0.0001
Others [no $t(9;22)$]	1.26 (1.02–1.55)	0.03
HLA		
Well matched	1.00	
Partially matched	1.44 (1.15–1.81)	0.002
Mismatched	1.37 (1.04–1.81)	0.02
Conditioning		
TBI regimens	1.00	
Non-TBI regimens	1.83 (1.28–2.62)	0.001
Relapse		
Cytogenetics		
Normal	1.00	
Hypodiploid, $t(4;11)$ or $t(8;14)$	2.45 (1.52–3.97)	<0.0001
Others [no $t(9;22)$]	1.26 (0.97–1.62)	0.08
Conditioning		
TBI regimens	1.00	
Non-TBI regimens	1.84 (1.19–2.84)	0.006
Source		
Related	1.00	
Unrelated BM	0.74 (0.56–0.97)	0.03
Cord blood (CB)	1.54 (1.09–2.17)	0.02
Non-relapse mortality		
Age at allo-SCT, year		
16≤, <45	1.00	
≥45	2.00 (1.47–2.73)	<0.0001
Cytogenetics		
Normal	1.00	
Hypodiploid, $t(4;11)$ or $t(8;14)$	1.98 (1.18–3.34)	0.01
Others [no $t(9;22)$]	1.14 (0.86–1.51)	0.37
HLA		
Well matched	1.00	
Partially matched	1.59 (1.16–2.17)	0.004
Mismatched	1.67 (1.16–2.41)	0.006
Conditioning		
TBI regimens	1.00	
Non-TBI regimens	1.99 (1.26–3.14)	0.003

Ph(−), ALL indicates Philadelphia chromosome-negative acute lymphoblastic leukemia; allo-SCT, allogeneic stem cell transplantation; TBI, total body irradiation.

preparative regimens were significant risk factors for relapse (Table 5).

The cumulative incidence of NRM was not statistically different among patients who underwent RD, URD, and CB allo-SCTs (39% in RD, 42% in URD, and 45% in CB at 3 years; $P = 0.17$). The results of multivariate analysis showed that ≥45 years of age at allo-SCT, cytogenetic abnormalities, HLA partially matched or mismatched, and non-TBI preparative regimens were significant risk factors for NRM (Table 5). Post-transplant lymphoproliferative disorder was observed in one patient, and there was no association with the use of ATG.

discussion

This report presents the results for the largest series of adult Ph(−) ALL patients who underwent allo-SCT. There were no significant differences between RD, URD, and CB allo-SCTs in any disease stage, suggesting that CB allo-SCT could be a treatment of choice for all disease stages of patients without a suitable RD or URD. There were no significant survival differences between BM and PBSC recipients in any disease stage (data not shown), which was consistent with other studies [27, 28]. Interestingly, OS after CB allo-SCT was significantly better than that after HLA-mismatched URD allo-SCT for CR1 patients younger than 45 years of age. These results might indicate advantages of CB allo-SCT when carried out for patients without an HLA-matched donor at an appropriate timing.

The major finding in this study is that OS was compatible between RD, URD, and CB allo-SCTs for Ph(−) ALL in CR1, even though NRM rates were higher in URD and CB allo-SCTs than RD allo-SCT. This is because of higher relapse rates in RD allo-SCT compared with URD and CB allo-SCTs. The low NRM rates due to the lower incidence of acute GVHD in our population might result from the differences in ethnic background [29, 30]. Although the NRM rates after allo-SCT in CR1 were not significantly different from those of URD allo-SCT in CR1, the causes of NRM would be different between URD and CB allo-SCTs. Hemorrhage due to insufficient platelet recovery and infection due to graft failure or delayed neutrophil recovery would be the major causes of NRM after CB allo-SCT in CR1. Since delayed engraftment is one of the most common limitations of CB allo-SCT [31–33], several attempts such as double cord units [34–36], intra-BM injection [37–39], and *ex vivo* expansion [40, 41] have been made to ensure engraftment. Although CD34-positive cell dose was not a significant risk factor for OS in this study, engraftment was delayed among patients who received fewer CD34-positive cells as previously reported [42]. Although all patients who underwent CB allo-SCT administered single CB intravenously, the technical progression of CB all-SCT could also improve the outcome of Ph(−) ALL as well as other hematological malignancies [21, 43–49] by reducing NRM.

Our results also indicated that CB allo-SCT beyond CR1 could achieve OS similar to that of RD or URD allo-SCT. It is noteworthy that some, but not all, patients with refractory disease could be rescued by CB allo-SCT as well as RD or URD allo-SCT [12]. Among patients transplanted in non-CR, survival of patients transplanted at ≥10 months from diagnosis was significantly superior to that of those transplanted <10 months from diagnosis (data not shown), suggesting that patients who could await a suitable donor or those with late relapse could obtain the advantages of allo-SCT. These patients could not have survived long with chemotherapy alone, and therefore, CB could be a hope of survival for patients with refractory disease who do not have a suitable RD or URD.

To our knowledge, this is the first and largest analysis of CB allo-SCT for Ph(−) ALL alone ($N = 233$). Recently, the results of a large retrospective analysis of a donor source that included data of 1525 patients (including 165 patients who underwent CB allo-SCT) were reported by the Center for International Blood and Marrow Transplant Research, the National Cord Blood Program, the European Group for Blood and Marrow Transplantation, and the Eurocord-Netcord registry [50]. The number of ALL patients who underwent CB allo-SCT was limited to 89 including both Ph(+) and Ph(−) ALL patients. The results of disease-specific analyses were also reported from Japan [CB; Ph(+): $N = 43$, Ph(−): $N = 71$] [51] and Minnesota (CB: $N = 69$) [52], with data of Ph(+) and Ph(−) ALL patients being analyzed together. Statistical techniques to adjust heterogeneities of the study population were used in those studies. Although we agree with the methodology of the above-described studies and the conclusions that support the use of CB for ALL patients without a suitable RD or URD, Ph(+) and Ph(−) ALL should be analyzed separately in an era of TKIs to obtain data which would be useful in clinical situations [9]. Our study clearly confirmed the usefulness of CB for Ph(−) ALL in any disease status.

In this type of retrospective study, selection biases from different backgrounds of patients who underwent RD, URD, and CB allo-SCTs could not be eliminated [12]. Considering that CB allo-SCT has not yet been recognized as a standard treatment of Ph(−) ALL, the background of CB recipients might be worse than that of other sources, that is, CB allo-SCT might be carried out for patients whose prognosis is considered to be poor without allo-SCT. In addition, since the median interval from diagnosis to allo-SCT in CR1 was similar between RD and CB allo-SCTs, the time-censoring effect, a major bias described elsewhere [12, 53, 54], did not affect our results. Although we could not make a comparison between chemotherapy and allo-SCT, our study could suggest promising data to broaden the choices of donor source.

In conclusion, the outcomes were comparable between RD, URD, and CB allo-SCTs in any disease status, and these may be considered equivalent options for patients with Ph(−) ALL. In the absence of a suitable RD or URD, CB allo-SCT should be planned promptly for Ph(−) ALL patients so as not to miss the appropriate timing.

acknowledgements

The authors thank Dr Seitaro Terakura for a thoughtful discussion.

funding

This study was supported in part by the Japan Leukemia Research Fund grant to SN, in part by the Research on Allergic Disease and Immunology (Health and Labor Science Research Grant), the Ministry of Health, Labor and Welfare of Japan to KM and YM, and in part by a Japanese Grant-in-Aid for Scientific Research to JT [no specific grant numbers].

disclosure

The authors have declared no conflicts of interest.

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VoxSanguinis
The International Journal of Transfusion Medicine

ISBT International Society of Blood Transfusion

Vox Sanguinis (2013) 104, 67–91

INTERNATIONAL FORUM

DOI: 10.1111/j.1423-0410.2012.01619.x

Vox Sanguinis

Donor selection and release criteria of cellular therapy products

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Within the last two decades, an increasing number of transfusion services worldwide significantly expanded their activities on the development and production of cellular therapy (CT) products, including the provision of cellular therapeutics for their use in clinical trials [1]. Due to the increasing importance of CT for Transfusion Medicine, the Editorial Board of *Vox Sanguinis* decided in 2007 to establish the new section 'Cellular Therapy' as the fifth section of the journal [2]. Within the first 5 years, a yearly increasing number of manuscripts have been submitted for publication. In addition, this expanding and innovative field of Transfusion Medicine very recently leads to the foundation of a joint ISBT-AABB Working Party (WP) on Cellular Therapies with the specific purpose of developing and sharing common views on the clinical use of cellular products between the ISBT and the AABB [3]. Furthermore, the WP activities will link together scientists already involved in CT projects from both sides of the Atlantic and other parts of the world. This will provide a platform for exchanging ideas and planning joint activities. It was agreed that a good starting point would be the development of consensus articles on donor selection and product release criteria.

The International Forum (IF) on these topics of CT was initiated by members of the above mentioned joint WP and provides an overview on current procedures based on different types of cellular therapeutics which are performed in well-experienced centres of six European countries (Austria, Finland, France, Germany, Italy, UK), Japan and the USA. The questions raised in this IF mainly focus on procedures for donor selection and final product release, both for products fulfilling the criteria of Advanced Therapy Medicinal Products (ATMP) following EU regulation 1394/2007/EC (or comparably manipulated cells), and for less engineered cellular therapeutics. Specifically, the following panel of questions were developed and submitted to cellular therapy experts of transfusion services and departments:

Question 1
What kind of CT products do you prepare in your centre?

Question 2
How do you select living donors based on the respective CT product prior to the collection of CT starting material?

Question 3
What assays do you perform to characterise the quality of the starting cell/tissue material?

Question 4
How do you perform quality control testing of the final CT product prior to release?

Question 5a
Have you defined and validated acceptable ranges/thresholds for the results of your release assays?

Question 5b
What is your policy if the acceptable ranges/thresholds of your release assays are not met?

Question 6a
Do you perform specific potency assays of the final CT product prior to release to the patient?

Question 6b
If potency assays are not performed prior to release: what kind of potency assays do you perform during the product validation phase of the respective CT product?

Each of the above questions should be answered with respect to the two principally different types of cellular therapy products:

Type I products: 'Engineered' cells or tissues (for example, 'Advanced Therapy Medicinal Products' following EU regulation 1394/2007/EC): Products containing cells or tissue that have been subject of substantial manipulation, or products containing cells or tissue that are not intended

to be used for the same essential function in the recipient as in the donor.

Type II products: Non-engineered cells or tissues (for example, 'Advanced Therapy Medicinal Products' which are not "engineered" according to definitions of EU regulation 1394/2007/EC, Annex I): Products containing cells or tissue that have only been subject to non-substantial manipulations, such as cutting, shaping, centrifugation, freezing, etc.

Since the legal framework may vary between countries, the definition of type I and type II products may also vary. Therefore the answers should take into account specific legal rules in the country concerned.

The authors describe various policies and procedures regarding the production of cellular therapeutics derived from different allogeneic and autologous sources, for example, umbilical cord blood, apheresis donations or harvests from bone-marrow and adipose tissue. The panel of provided CT products cover a wide range of clinical indications, such as the use of mesenchymal stromal cells (MSC) in regenerative medicine (for example, in cardiac regeneration or treatment of critical ischemias), dendritic cells as anti-cancer vaccines or immuno-therapeutic approaches with MSC, chimeric T cells and cytokine-induced killer cells. Furthermore, valuable information is given about the different panel for quality control assays and potency assays to qualify the final CT product prior to release for clinical application.

In parallel to the mostly highly complex procedures linked to the development, processing and validation of innovative cellular therapeutics, the legal framework in different parts of the world seems to be at least as complex. Even in the European Union with its partly supra-national legislation defined by directives, regulations and guidelines, the kind of implementation of such rules in national law of the different member states and the respective responsibilities of national authorities varies significantly. The responses to some of the questions of the IF are pointing out the complexity and diversity of current regulations, and this situation might negatively influence the implementation of international clinical trials using CT products. Nevertheless, the results of this International Forum discussion are a further proof that the development, production and provision of ATMPs and other CT products will inceasingly influence and modify the scientific activities of at least academic transfusion services.

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K. Schallmoser & D. Strunk

Question 1

Type I products: engineered cells or tissues ATMP. We isolate and prepare bone marrow (BM)-derived mesenchymal stem/progenitor cells (MSPCs) in an animal component-free culture system with minimal manipulation steps for clinical application [1]. The use of human platelet-derived growth factors has been efficiently established for routine MSPC expansion protocols [2]. MSPCs are considered to be immune regulatory therapeutics in graft-versus-host disease (GvHD) and for tissue repair/bone regeneration in critical size bone defects. Additional experimental procedures have been implemented for the

preclinical propagation of endothelial colony-forming progenitor cells (ECFCs) from the peripheral blood of healthy volunteers and patients suffering from various cardiovascular diseases [3]. ECFCs will be tested in clinical trials for their safety and feasibility in realizing therapeutic vasculogenesis. Additional experimental procedures are available to generate off-the-shelf multicellular products comprising autologous (to each other) MSPC + ECFC pairs which are propagated from the same umbilical cord and cryopreserved in conjunction with the corresponding umbilical cord blood [http://www.jove.com/video/1525/isolation-and-animal-serum-free-expansion-of-human-umbilical-cord-derived-mesenchymal-stromal-cells-mscs-and-endothelial-colony-forming-progenitor-cells-ECFCs].

Type II products: non-engineered cells or tissues. Preclinical production of human lineage-negative cell fractions from non-mobilized peripheral blood has been established based on CD2/CD19/CD56 depletion [4].

The following questions Q2–Q6B were answered regarding the CT products indicated in Q1:

Question 2

Donors have to fulfill the criteria according to the Austrian legal regulations based on European law [Directive 2004/23/EG of the European Parliament and Council, 31 March 2004 (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32004L0023:DE:HTML)] and are further selected with the aim of reaching a minimized risk for the donor and the recipient of the cell therapeutics.

Detailed medical history and physical examination as well as appropriate laboratory tests are acquired at maximum 7 days prior to the stem cell collection. These comprise exclusion of infections by treponema pallidum, hepatitis B virus (HBV), hepatitis C (HCV), hepatitis A (HAV), cytomegalovirus (CMV), parvovirus B19 and human immunodeficiency virus (HIV) by syphilis screening (ELISA or TPHA test), testing for HBs-Ag, anti-HBs, anti-HBc, anti-HCV, anti-CMV and anti-HIV-1,2 antibodies, and nucleic acid-based tests (NAT) for HBV, HCV, HAV, parvovirus B19 and HIV, respectively. Infection by the human T-lymphotropic virus type I (HTLV-1) is only excluded by anti-HTLV-1-antibody testing if donors or their parents and sexual partners are living in or hail from regions with high incidence. The medical qualification for BM puncture depends furthermore on a normal full blood cell count and normal coagulation parameters, and absence of renal or hepatic diseases.

Additionally, donors are routinely tested for ABO blood groups and irregular erythrocyte allo- and autoantibodies. They receive detailed information regarding possible side effects and risks of the procedure and have to give informed consent. All diagnostic findings and relevant documents are collected, signed by the responsible physician, and saved in the patient history form for at least 30 years. The identity of the donor is confirmed again immediately before the intervention. For the application of MSPCs for bone regeneration, autologous cell therapeutics are preferred in our centre. In the immune modulatory setting treating GvHD, we use allogeneic donors (either third party or, if available, the identical donor of the haematopoietic stem and progenitor cell (HSPC) transplant).

Question 3

Quality assays as identity, purity, potency, sterility or suitability, if applicable, methods used. In our centre, BM is harvested for non-haematopoietic stem cell therapy in small aliquots of 3–5 ml per aspiration site with a maximum of 6–8 aspirations. The volume of the collected BM aspirate is determined after pooling, and a 1-ml aliquot is taken sterilely for further analyses. Then, the BM aspirate is seeded without further manipulations in culture medium supplemented with pooled human platelet lysate. Microbiological testing to exclude fungal, anaerobic and aerobic bacterial contamination is performed by an automated blood culture system (BacT/Alert, bioMérieux SA, Marcy l'Etoile, France). The leucocyte count is analysed by an automated blood cell analyzer (ADVIA 2120i, Siemens Healthcare Diagnostics GmbH, Vienna, Austria). The concentration of viable mononuclear cells (MNCs) is estimated by flow cytometry analysing the percentage of 7-amino-actinomycin D $(7\text{-AAD})^-/CD45^+/$ glycophorin A^- cells within a mononuclear cell forward and rectangular light scatter region. In parallel to the primary culture, the clonogenicity of BM-derived MSPCs is tested by colony-forming unit of fibroblast (CFU-F) assays in fivefold serial diluted seeding densities of MNCs. Quality thresholds for CFU-F frequency have not yet been established.

Question 4

In process controls. Manipulation steps during cell culture are reduced to a minimum as previously described [5]. In brief, 2–3 ml of heparinized BM is regularly re-suspended in 500 ml culture medium before seeding in one four-layered cell factory comprising 2,528 cm^2 of culture area. At day three, non-adherent cells are removed by vigorously washing with 37°C pre-warmed phosphate-buffered saline (PBS) before adding new supplemented medium. Continuous growth factor support is guaranteed by replacing 20% of the medium twice weekly with new animal protein-free medium supplemented with pooled human platelet lysate [http://www.jove.com/video/1523/

preparation-of-pooled-human-platelet-lysate-phpl-as-an-efficient-supplement-for-animal-serum-free-human-stem-cell-cultures]. Continuous visual microscopic control and sterility testing of the conditioned as well as the fresh culture medium at each medium exchange is performed to detect a bacterial or fungal contamination as soon as possible.

After the final trypsinization step, a 1-ml aliquot of the harvested MSPCs is taken sterilely for the following quality controls and the product lot release:

Cell number and viability testing. The number of MSPCs generated in one culture passage is determined using a haemocytometer as the mean of four measurements. Viability is determined by trypan blue exclusion in the haemocytometer and 7-AAD analysis by flow cytometry, respectively.

Analysis of immune phenotype. Being aware of the heterogeneous mixture of cultured MSPC populations, we define the MSPC purity as >90% reactivity for CD73, CD90, CD105 and MHC class I and negative in >98% for CD3, CD14, CD19, CD31 and CD34. Haematopoietic cell contamination as defined by total CD45 reactivity is required to be below 10% and MHC class II reactivity of MSPCs must not exceed 10% [5]. Modified limits for $CD3^+$ T cell contamination are applied in the event that haploidentical transplantation adheres to the maximal T cell dose defined by the corresponding HSPC transplantation protocol.

Sterility testing. The final MSPC product is again tested for fungal, anaerobic and aerobic bacterial contamination by automated blood culture (BacT/Alert). A potential mycoplasma contamination is excluded using a bioluminescent reporter assay for mycoplasma-specific enzymes (MycoAlert® Lonza Basel, Switzerland) [1].

Endotoxin. As an alternative for pyrogenicity testing, endotoxin measurement is performed by a quantitative chromogenic Limulus Amebocyte Lysate (LAL) assay (Endosafe®, Charles River Laboratories International, Wilmington, MA). The LAL assay enables the microbiologic release of the cell product within two hours of harvest to circumvent the delay of testing by bacterial culture methods. According to the Food and Drug Administration (FDA) recommendations, our release criteria require endotoxin levels below 5 EU/kg recipient body weight per dose [5].

Genomic stability. Serious concerns have arisen about the occurrence of hazardous genomic aberrations in *ex vivo* expanded MSPCs. We have demonstrated genomic stability by array-comparative genomic hybridization (aCGH) analysis of clinical scale-cultured MSPCs and in association with replicative senescence without evidence for malignant transformation [1, 6]. A standardized attitude for the routine product lot release is not yet defined.

Question 5

Cell dose. Until now, there has been no standardized culture protocol and no general recommendation defined for the optimal dose of MSPCs due to the lack of appropriate dose finding clinical studies. Due to a pragmatic approach, we generate as many MSPCs in the shortest culture duration with the fewest manipulation steps possible according to our efficient cell expansion protocol. Getting a cell number of, for example, more than 8×10^8 MSPCs as routinely generated in eight four-layered cell factories on 20 224 cm^2 (Thermo Fisher, Nunc International, Naperville, IL, USA) enables us to treat an adult patient of 80 kg body weight with a maximum 10 million of MSPCs per kg body weight. Whether a single high dose i.v. infusion or a serial therapy with respective lower cell yields is more efficient as well as the optimal cell dose locally injected for tissue repair still have to be evaluated systematically in ongoing clinical trials. Based on the efficiency of our protocol, we lack experience with not meeting a lower threshold of 2 million of MSPCs/kg. Our release criteria define an additional passage in case the required cell dose was not reached. The optimal cell dose for local application (i.e. for bone repair purposes) is currently determined in animal experiments but also needs to be tested in dose finding studies.

Viability testing. For clinical application, the viability of MSPCs after trypsinization and after cryopreservation is required to be >90% in our release criteria. Only in justified cases may a reduced viability of >70% be permissible. Whenever possible we require an additional short culture passage to be performed to enrich the viable cells and to deplete the dead cell fraction by removing non-adherent cells and debris by a complete medium change 24 h after re-seeding. Furthermore, the conditions of the culture procedure should be re-assessed to identify possibilities for process optimization.

Analysis of immune phenotype. Not meeting the MSPC purity as defined by immune phenotype marker profiling may indicate either a putative contamination with haematopoietic BM-derived cells or otherwise a possible activation state of MSPCs as, for example, reflected by an elevated expression of MHC class II antigens. A further culture passage should be regularly performed to further enrich MSPCs and reduce contaminating haematopoietic cells. For further culture procedures, an optimization of the washing step removing the non-adherent cell fraction at the beginning of the primary culture passage should be executed. The clinical relevance of elevated MHC class II expression in

particular on third party MSPCs is not yet clear and should be considered with caution. Also in this case, an additional culture passage and re-testing is recommended in our protocol.

Sterility testing and endotoxin. In our standard culture procedure, we avoid the use of antibiotics/antimycotics to detect any contamination as early as possible and to prevent patient immunization or exacerbation of pre-existing allergies to common antibiotics. Any sign or proof of a microbiological contamination of the cell product leads to the complete disposal of the product and requires decontamination of the laboratory equipment in our practice. For safety reasons, we perform an antibiotic/antimycotic reserve culture procedure in parallel in two cell factories to create a backup cell product if the standard MSPC product has to be discarded. In the event of negative microbiological testing of the standard product, this additional MSPC product is used for research purposes.

Question 6

Potency assays for MSPCs are currently not performed prior to the release of MSPCs in our centre for GvHD therapy. We are currently underway towards the establishment of appropriate protocols testing the capacity for osteogenic and chondrogenic differentiation and for the support of angiogenesis of MSPCs and ECFCs *in vitro* and in immune-deficient mice *in vivo* as well as their immune modulatory effect *in vitro*.

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J. Nystedt, T. Kaartinen & M. Korhonen

Question 1

Type I products: The Advanced Cell Therapy Centre of the Finnish Red Cross Blood Service (FRCBS) currently prepares allogeneic bone marrow-derived mesenchymal stromal cells (BMMSCs) expanded in platelet-rich plasma for acute steroid-resistant GvHD.

Type II products: The FRCBS also maintain a FACT/NetCord accredited cord blood bank (the Finnish Cord Blood Bank) and a Bone Marrow Donor Registry with 20 000 prospective donors. However, we will only focus on the type I products in the subsequent questions.

Question 2

Donor eligibility is assessed by a donor eligibility questionnaire dealing with questions about common health status, possible long-term or severe diseases, previous blood transfusions, sexual behaviour, travelling, possible contraindications for the procedure (heart-disease, local anaesthesia), other risk behaviour for possible infectious diseases (tattoos, etc.), current skin diseases/infections, family history of inherited diseases.

The donors are tested for the following infectious diseases:

NAT tests: HCV-RNA, HIV-RNA, HBV-DNA, B19-DNA (Parvo), HAV-RNA.

Serologic tests: HBsAg, Anti-HCV, Anti-HIV (1+2), Anti-Hbc, Syphilis, HTLVI/II, S-CMVAb.

The blood samples for the donor screening are drawn within 30 days of the CT starting material collection. Additionally, the ABO blood group is determined and in intermediate resolution HLA screening is performed.

Table 1 Batch release of BMMSCs

Quality assay	Method	Release criteria
MSC phenotype	Flow cytometry (validated)	≥95% : CD44, CD49e, CD13, CD90, CD73, CD29, CD105 ≤2%: CD14, CD19, CD34, CD45 FYI: HLA-ABC, HLA-DR
Viability	NucleoCounter automatic cell counting system (propidium iodide-based) (validated)	≥90% before freezing
Osteogenic differentiation	*in vitro* diff. assay	+ (FYI)
Adipogenic differentiation	*in vitro* diff. assay	+ (FYI)
Sterility testing	BacT/ALERT 3D aerobe and anaerobe growth (validated)	No growth
Endotoxin levels	LAL assay (validated)	Less than 5 IU/kg patient weight
Cell morphology	Phase-contrast microscopy	Normal
Karyotype	G-band analysis (validated)	46, XX/XY
Population doubling number	Cell yield starting from CFU-F number	≤30

Question 3

The quality of the bone marrow aspirate is analysed with the following methods:

(1) a differential blood cell count is performed with a Sysmex XE-2100 haematology analyzer
(2) sterility testing: aerobic and anaerobic bacterial growth with the BacT/Alert 3D system
(3) stromal cell content using the CFU-F assay

Question 4

Every MSC batch will be tested as follows (Table 1):

Question 5a

Defined yes. Critical assays have been validated (MSC phenotype characterization, viability, sterility testing). Ranges have been defined using results from process development and literature.

Question 5b

The batch will not be released for clinical use. Differentiation potential results are for information only, but will be routinely performed.

Questions 6a and 6b

An *in vitro* immunosuppression assay is performed, but the results are not release criteria (only for information). An in-house developed flow cytometry-based assay measuring inhibition of activated PB-MNCs is used.

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S. Fleury-Cappellesso & L. Sensebé

Question 1

Our centre is located at the French National Blood Services (Etablissement Français du Sang: EFS) in Toulouse. Autologous and allogeneic haematopoietic stem cells (HSC), from peripheral blood (PBSC) or bone marrow (BM), are routinely prepared as type II products.

Since 2006, EFS is involved in clinical trials using mesenchymal stromal cells: (i) for regenerative medicine using autologous bone marrow mesenchymal stem cells (BM-MSC) or adipose tissue-derived stromal cells (ACS) to treat, respectively, cardiac insufficiency and critical limb ischemia; (ii) for treating GHVD using allogeneic BM MSC. These productions could be defined as type I products according to EU regulation 1394/2007/EC.

Question 2

Whether type I or type II products, the donor is always evaluated for potential risks related to the collection procedure by a licensed physician. An informed consent from the donor shall be obtained prior to the collection. Donation, procurement and testing are performed in compliance with French Public Health Code. Donors are evaluated for risks factors for disease transmission by medical history, physical examination and laboratory testing. Within 30 days prior to collection, donors are tested for infection disease agents mentioned in article R.1211-14, R.1211-15 and R.1211-16 of the Public Health Code:

(1) Human immunodeficiency virus, type 1 and type 2 (HIV-1 and HIV-2)
(2) Hepatitis B virus
(3) Hepatitis C virus
(4) Syphilis
(5) Human T cell lymphotrophic virus I
(6) Cytomegalovirus

Depending on donor's history, others tests could be performed as toxoplasma, EBV, West Nile Virus and *Trypanosoma cruzi* (Chagas' disease). All testing procedures are performed in accredited laboratories authorized by regulatory authorities. Identity of the donor is checked by a physician prior to the collection and includes the name, date of birth, age, gender and medical history.

Type I products. Donors are carefully selected according to inclusion and exclusion criteria defined in the clinical study protocol. These criteria should be approved by an ethic committee and French regulation authority before the clinical trial began. For allogeneic uses of BM-MSC and ASC, HLA A, B, C and DR typing is done and could be used.

Type II products. Donors selection and evaluation are performed according to JACIE. For collection of HSC by apheresis, an evaluation of venous statement, a possible need for central venous access and the mobilization therapy is evaluated within 2 weeks prior to the collection. For bone marrow collection, an evaluation of potential risks of general anaesthesia is performed within 30 days.

In allogeneic context, the donor eligibility is determined by a physician after medical history, exam and testing before the recipient's high dose therapy is initiated and before the donor begins mobilization regimen. Allogeneic donor eligibility is determined regarding HLA compatibility with the recipient, serologic status and the potential risk of the collection. HLA typing is performed by a laboratory accredited by EFI and includes HLA-A, B, C and DR type.

Allogeneic donors are also tested for red cell compatibility with the recipient.

Question 3

At the arrival, cell engineering unit verifies the integrity of the packaging, aspect of the product, duration and temperature of the transport, identity of the donor and coherence with accompanying documents.

Type I products. The safety of the clinical trial using BM-MSC & ASC is directly linked to specific controls implemented during the cell processing and depend on starting tissue material [1].

For ASC production, fat tissue weight, cell counting, viability, phenotype analysis and sterility control are carried out [2]. For BM-MSC, bone marrow volume, cell counting, viability, clonogenic potency (CFU-F) and sterility control are performed. For recipient safety, sterility control must be negative.

Type II products. Evaluation of cellular therapy products is based on a total nucleated cell count, viability, CD34+ cells enumeration [3], CFU-GM assay and sterility control.

Question 4

Quality assays and release criteria have to be suitable and compatible with a rapid release of the graft. For this purpose, in some cases, quality control results of previous steps of the processing must be taken into account to release the final product.

Type I products. Quality controls and release criteria have been validated and defined in the clinical study protocol. Final controls for ACS and BM-MSC production include cell counting, viability, phenotype analysis and bacteriological control.

In France, the genetic stability of cultured MSC is checked by karyotyping 30 metaphases. The karyotype is only used as release control when cultured BM-MSC & ASC are cryopreserved and used after thawing. Moreover, MSC being not prone to transformation and karyotype not being completely relevant, we are testing other potential markers as hTERT expression and Q-PCR for different gene products (e.g. p53, p21 & c-myc) [4].

Type II products. Haematopoietic stem cells are released only in view of a nominative prescription. Autologous grafts are validated in the light of quality controls before freezing and include CD34 cell count, viability and bacteriological test. Beside these tests, ABO incompatible allogeneic grafts are evaluated for red blood cell volume.

Questions 5a and 5b

Sterility control of the last stage of the process is known in posteriori because of the time of culture (12 days according to French regulation recommendations). Therefore, sterility control is evaluated at a previous stage, if applicable, and must be negative.

Type I products. Phenotypic criteria for release MSC has been validated on more than 80% of mesenchymal markers expression (for ASC: CD90, CD73 and CD13; for BM-MSC: CD90 andCD73) and the lack of haematopoietic cells (CD45 and CD14 < 5%) [5].

In our centre, MSC are produced for phase I clinical trials. Viability threshold should be more than 70% and more than 75% for, respectively, ASC and BM-MSC production. The cell number should be in accordance with the predefined dose (e.g. 100×10^6 for ASC in limb ischaemia and 80×10^6 BM-MSC in cardiac ischaemia).

Type II products. After conditioning regimen, the minimum dose to achieve acceptable engraftment is 2×10^6 CD34/kg. In case of insufficient dose of CD34+, the final product is released but may induce a delay in haematopoietic recovery. The final decision is always subject to the physician approval for use regarding the urgent medical

need and the absence of therapeutic alternative. In the presence of ABO-incompatibility, separation using density gradient (BM graft) or dilution (PBSC graft), residual red blood cell volume is decreased under 15 ml.

Questions 6a and 6b

Potency assays are not performed prior to release. During the validation phase of processes developed for production of BM-MSC and ASC, two types of potency assays are implemented:

(1) For immunosuppressive activity: using immunological platform developed by INSERM unit U917 for two programs of 7th FP of EC (CASCADE n°223236 and REBORNE n°241879), the mechanisms and effects of MSC (BM MSC & ASC) on effectors of innate and adaptative immunity are systematically tested.
(2) For uses in regenerative medicine, the following assays are used: (i) in bone repair, potential osteoblastic differentiation using our previously reported differentiating method [6], (ii) for vascular repair purposes, dosage of some released cytokines and growth factor (e.g. VEGF).

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Question 1

Type I products (all ATMPs).

(1) Autologous bone marrow mononuclear cells for cardiovascular regeneration.
(2) Mesenchymal stroma cells (third party, mixed from several unselected donors).
(3) Cytokine-induced killer cells (CIK) (allogeneic stem cell donor derived).

Type II products (non-ATMP cellular therapeutics).

(1) Haematopoietic stem and progenitor cells from marrow (also ery-depleted, volume-reduced, washed), mobilized peripheral blood (also volume-reduced, washed) and cord blood (ery-depleted and volume-reduced) for haematopoietic reconstitution, autologous and allogeneic, also cryopreserved – 'standard' cellular therapeutics, not addressed here.
(2) Matched-donor lymphocytes (DLI), also cryopreserved – 'standard' cellular therapeutics, not addressed here.
(3) Haematopoietic stem and progenitor cells from mobilized peripheral blood, autologous and allogeneic, immunomagnetically manipulated (CD34+ selected, CD3/CD19 depleted), also cryopreserved (unmatched-related or unmatched-unrelated).
(4) Antigen-specific matched-donor or unmatched-donor lymphocytes, immunomagnetically manipulated (Adenovirus, Cytomegalovirus), also cryopreserved.
(5) Femoral head, allogeneic, heat-inactivated, cryopreserved.

Question 2

Type I products.

(1) Autologous bone marrow mononuclear cells for cardiovascular regeneration: Marrow is drawn by treating physician; treating physician does donor assessment with regard to donor safety and indication for cellular therapy, as well as written informed consent. The treating physician fills the collection form and peels unique labels from the collection form, which he attaches to the cellular product as well as to companion blood tubes; he confirms donor identity with his signature on the collection form. IDMs do not play a role in donor eligibility testing for this product.

(2) Mesenchymal stroma cells, third party: Donors are invariably bone marrow donors for 'bone marrow transplantation' from whom we draw an extra aliquot of marrow with their written informed consent and approval by the IRB. Thus, donor eligibility, including IDMs, and donor ID are the same as for marrow donors (acc. to German Stem Cell Guidelines, German Transfusion Law, WMDA standards).

(3) CIK cells: Donors are evaluated as DLI donors, according to national and international guidelines, including eligibility, IDMs, donor ID, etc.

Type II products.

(1) Immunomagnetically manipulated stem cell products: Donor eligibility evaluation is done according to national and international guidelines for stem cell donors, including IDMs, donor ID, etc.

(2) Immunomagnetically manipulated T-cell products: Donor eligibility evaluation is done according to international guidelines for stem cell donors, including IDMs, donor ID, etc. The only additional test is for the frequency of antigen-specific T-cells. Whenever possible (contingent on the presence of at least 1:1000 antigen-specific T-cells), the original stem cell donor is used. If not possible, a reasonably matched (at least haplo-identical) third-party donor is selected from a stem cell donor registry.

(3) Femoral head: Donor eligibility assessment is done by orthopaedic surgeons based solely on donor history. Written informed consent is obtained by the same surgeon. No IDMs are tested prior to donor clearance. The surgeon who removes the femoral head fills the collection form and peels unique labels from the collection form, which he attaches to the product container as well as to companion blood tubes and informed consent forms; he confirms donor ID with his signature on the collection form.

Question 3

Type I products.

(1) BM-MNCs for cardiovascular regeneration: Processing center does serology for Hepatitis A, B, C, HIV, Syphilis on blood. Incoming material is tested for integrity, completeness, CBC (automated haemocytometer), sterility (culture – pending at the time of release).

(2) Mesenchymal stroma cells: Starting material comes with all the pertinent test results for allogeneic BM for stem cell transplantation, including IDMs (because the companion product is cleared and released for transplantation). Incoming BM specimen is additionally tested for CMV genome in the cell pellet as required by the federal overseeing body. Sterility is tested by culture: CBC, volume, total cell number, total MNC number as above.

(3) CIK cells: Volume, CBC, sterility, CD3, CD25, CD56; total cell count is calculated from these data. Day of collection IDMs (serology and NAT).

Type II products.

(1) Immunomagnetically manipulated stem cell products: Starting material is a cleared stem cell product; all the pertinent quality-defining values are available [CBC, viability (7AAD), CD34+, CD3+, Hkt, Volume, IDMs]. No additional quality tests are required.

(2) Immunomagnetically manipulated T-cell products: Starting material is a cleared DLI product – see above. In addition, the frequency of antigen-specific T-cells is again assessed, but values are not available until the end of processing and only serve for quality control purposes (recovery).

(3) Femoral head: Availability of all paperwork and blood for serology and NAT, integrity of the container, proper labelling of all papers, blood tubes and container with unique product number.

Question 4

Type I products.

(1) BM-MNC for cardiovascular regeneration: Outgoing material is tested for CBC (specification: 5–50 × 10E7 TNC, Leuko > 70%), sterility (culture – pending at the time of release). We do not have a potency assay, because we have not identified a non-clinical surrogate potency marker. Not required for release are analyses of *in vitro* migratory potential, CD34, CD14, KDR, CD133, CXCR4 and some other surface markers which are routinely performed, by virtue of which we are hoping, in the future, to identify predictive 'potency markers' for our cell product.

(2) MSCs: Release of MSC products is contingent on the qualification and clearance of the 'master cell bank' from an aliquot of which the clinical product was generated. Clearance of the master cell bank is very complex (including MLR, senescence, freedom from a large panel of viruses and other pathogens, identity through FACS and functional assays) and detailed description of the process exceeds the limitations of this article. The aliquots for clinical use are cryopreserved. They are released based on sterility, as well as FACS analysis prior to cryopreservation for viability by dye exclusion (>90%), CD45, CD14, HLA-DR (all <10%) and CD90, CD73, CD105 (all >90%) and freedom from endotoxin.
(3) CIK cells: Release requires testing for CD3, CD25 and CD56, sterility of a sample drawn 2 days prior to the harvest at the time of release (culture results pending), and a >10-fold increase of the frequency of CD3+CD56+ cells. An aliquot containing a number of potentially allo-reactive T-cells (CD3+CD56−) as defined by the treating physician is released non-irradiated, the remainder is released after irradiation with 3000 cGy.

Type II products.

(1) Immunomagnetically manipulated stem cell products: Measurement of total cellularity, CD34+ cells (CD34-enriched products: >90%, otherwise 'declared') and CD3+ cells (declared), viability (>70%). In CD3/CD19 depleted cell products, additionally residual B-cells and NK-cells are measured. The number of allowable T-cells is defined by the clinician (in writing). Potentially, less than the entire product is released to the patient. The remainder can be cryopreserved for later use. Sterility is tested (culture), but the results will not be available upon release.
(2) Immunomagnetically manipulated T-cell products: Measurement of total cellularity, CD3/4/8+ cells, and % antigen-specific T-cells (by IFN-release after antigen-stimulation or by streptamer technology, depending on the clinical trial; release criterion: >50% purity). The total number of allowable potentially allo-reactive (not antigen-specific) T-cells is defined by the clinician (in writing). Potentially, less than the entire product is released to the patient (dosing based on allo-reactive T-cell contents), the remainder can be cryopreserved for later use. Sterility is tested (culture), but results will not be available upon release.
(3) Femoral head: IDMs negative for Hepatitis A, B, C, HIV, Syphilis by serology, Hepatitis B, C and HIV by NAT, supernatant sterile, all documents available.

Question 5a

Type I and type II products. Yes – see above.

Question 5b

For all products: Per specification, only 90% of products must meet release criteria. When they do not, for all DIRECTED products, clearance and release is possible if the treating physician declares the vital indication in writing. This specifically also applies to autologous products positive for any type of infectious agents, including HIV and Hepatitis, as well as to non-sterile products. Non-directed products (femoral head, MSC) that do not meet release criteria are discarded or may be used for laboratory research.

Question 6a

Type I products.

(1) BM-MNCs for cardiovascular regeneration: No, we have not been able to identify a surrogate marker of potency.
(2) MSCs: Only during validation of the master cell bank, suppression of a mixed lymphocyte reaction is tested and is requisite for clearance of the master cell bank.
(3) CIK cells: We consider the emergence of CD3+CD56+ cells a potency assay. Direct assays are not performed prior to release.

Type II products.

(1) Immunomagnetically manipulated stem cell products: The number of CD34+ cells/kgBW of the recipient is used as potency assays.
(2) Immunomagnetically manipulated T-cell products: detection of IFN-secreting or antigen-binding cells, respectively, is considered potency assays of antigen-specific T-cell products. The accuracy of this assumption, except for some evidence of a dose–response relationship *in vitro* and in a xenograft model, has not been directly assessed.
(3) Femoral head: No, we do not know of such an assay.

Question 6b

Type I products.

(1) BM-MNCs for cardiovascular regeneration: After evidence of its potency in mice, a clinical trial demonstrated the clinical potency of products generated according to our method – notably for the cohort, not for every individual. Markers that can predict potency of a given product have not been identified.

(2) MSCs: Suppression of MLR – see above. Markers that can predict potency of a given product have not been identified.
(3) CIK cells: Suppression of tumour and leukaemia cells in a xenotransplant assay. Markers that can predict potency of a given product have not been identified.

Type II products.

(1) Immunomagnetically manipulated stem cell products: Clinical trials support the use of CD34+ cell dose as a potency assay.
(2) Immunomagnetically manipulated T-cell products: It is not clear whether there is a dose–effect correlation for antigen-specific T-cells, since they proliferate *in vivo* in response to antigen contact. It is reasonable to assume, however, that a larger allo-reactive T-cell dose bears the risk of GvHD.
(3) Femoral head: Sufficient evidence abounds about the clinical potency of heat-inactivated allogeneic unmatched bone grafting using the Telos-method. No additional evidence was sought, nor was any requested by the regulators.

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Question 1

The Center of Transfusion Medicine, Cellular Therapy and Cryobiology (CTMC) includes (i) a public cord blood bank named 'Milano Cord Blood Bank', (ii) a laboratory in charge of processing haemopoietic progenitor cells from bone marrow (HPC-Marrow), HPC from mobilized peripheral blood (HPC-Apheresis) and allogeneic Lymphocyte-Apheresis units, (iii) a GMP laboratory for the preparation of advanced therapy medicinal products (ATMP) in compliance with EU regulation 1394/2007 and (iv) a flow cytometry laboratory performing cell immunophenotyping.

During February 1993 – September 2011, the Milano Cord Blood Bank, which was accredited by the Foundation for the Accreditation of Cellular Therapy (FACT) in 2004, has received and cryopreserved 27 562 and 9465 cord blood units (HPC-CB) from 37 delivery suites, respectively. From the cryopreserved inventory, 467 units (4·9%) have been distributed for unrelated HPC transplantation to 146 transplant centres worldwide. In addition, the bank has stored 461 directed HPC-CB units from families with a relative affected by a condition with an indication to family-related CB transplant. Seventeen (3·7%) of the latter units have been transplanted. On 18 August 2011, the Milano Cord Blood Bank has qualified to participate under the US National Marrow Donor Program (NMDP) Investigational New Drug (IND) application: A Centralized Cord Blood Registry to Facilitate Allogeneic, Unrelated Donor Umbilical Cord Blood Transplantation, effective 20 October 2011. The Milano Cord Blood bank does not perform commercial autologous CB storage. HPC-CB units donated to the Milano Cord Blood Bank are processed with a volume reduction procedure using a Compomat G4 device (Fresenius) [1].

In addition to managing the public HPC-CB allogeneic inventory, the staff of the Milano Cord Blood Bank performs the characterization, cryopreservation and thawing of HPC-Marrow, HPC-Apheresis and Lymphocytes-Apheresis units collected for clinical use in the hospital HPC transplant program, which was accredited in 2010 by the Joint Accreditation Committee-ISCT (Europe) & EBMT (JACIE). During 2010, a total of 64, 33 and 91 HPC-Marrow or HPC-Apheresis units have been cryopreserved, minimally manipulated and thawed, respectively.

The GMP laboratory incharge of ATMP preparation – named 'Franco Calori Cell Factory' – develops experimental products and clinical trials of cellular therapy in cooperation with clinicians of different specialties including haematology, cardiology, hepatology, neurology, orthopaedics, nephrology and neonatology. Since the onset of activities in 2000, the Franco Calori Cell Factory, which was accredited by the Italian Drug Agency (Agenzia Italiana del Farmaco, AIFA) in 2007, has prepared 50 units of ATMP, which have been distributed to five clinical centres for use in five experimental protocols. In addition, approximately twice as many ATMP have been prepared for process validation purposes.

In summary, we prepare the following type I products (i.e. those with substantial manipulations or not intended to be used for the same essential function in the recipient as in the donor): (i) expanded CD34+ cells from HPC-CB purified with the CliniMACS device (Miltenyi, Bergisch Gladbach, Germany) [2]; (ii) CD133+ cells from HPC-Marrow and HPC-Apheresis, purified with the CliniMACS device; (iii) autologous and allogeneic mesenchymal stem cells from adipose tissue (MSC-AT), bone marrow (MSC-Marrow) and CB (MSC-CB), purified with adherence procedures and *ex-vivo* expanded with different protocols.

Moreover, we prepare the following type II products (non-substantial manipulations): (i) HPC-CB for allogeneic transplant; (ii) autologous and allogeneic HPC-Marrow; (iii) autologous and allogeneic HPC-Apheresis; (iv) allogeneic Lymphocytes-Apheresis units.

Table 1 Cord blood testing

Test	Time	Sample
Collected nucleated cell count	Banking	Whole blood withdrawn from collection bag
Cryopreserved nucleated cell count	Banking	Buffy coat after volume reduction
Nucleated cell count	Before unit release	Segment attached to the frozen bag
Nucleated red cell count	Banking	Blood film from buffy coat.
ABO and Rh blood type	Banking	Red cell fraction after volume reduction
Genomic HLA-A,B low resolution and DRB1 high resolution typing	Banking	Segment from the frozen bag detached before freezing
Genomic HLA-A,B high resolution and DRB1 high resolution typing if not performed before (old units). Other HLA loci at requested level of resolution	Upon request when the unit is selected for a patient	DNA extracted from segment attached to the frozen bag
Confirmatory HLA-A,B,DRB1 typing	Before unit release	As above
Maternal HLA-A,B,DRB1 typing	Before unit release	DNA extracted from repository of samples, collected at 6 months check or, if absent, at delivery (EDTA sample)
CD34 + cell count	Banking	Buffy coat after volume reduction
Haemoglobinopathy screening	Banking	Whole blood from collection bag
Cell viability determined if transport temperature is out of range	Banking	Whole blood from buffy-coat bag
Cell viability	Before unit release	Segment attached to frozen bag
Clonogenic potential evaluation: CFU-GM, CFU-GEMM, BFU-E, CFC	Banking	Buffy coat after volume reduction
Clonogenic potential evaluation: CFU-GM, CFU-GEMM, BFU-E, CFC	Before unit release	Segment attached to frozen bag
Microbial culture for aerobic and anaerobic bacteria and fungi	Banking	Unit sample at end of processing prior to cryopreservation, after cryopreservative addition
HBsAg, anti-HCV, anti-HIV 1-2, HCV/HIV1 RNA - HBV DNA NAT and anti-Treponema P. test	Banking	Maternal serum collected at delivery
Anti-HBc (total and IgM) total and IgM, ALT, anti-CMV IgG and IgM and anti-Toxoplasma IgG and IgM test, anti-HTLV I-II	Unit selection for a patient	Maternal serum collected at delivery
HBsAg, anti-HCV, anti-HIV 1-2, and anti-Treponema P. test	Six months check after delivery	Maternal serum sample collected 6 months after delivery
ALT, anti-CMV IgG and IgM and anti-Toxoplasma IgG and IgM test, anti-HTLV I-II	Before unit release	As above
HBsAg, anti-HIV 1-2, anti-HCV, anti-HTLV I-II test	Before unit release	Unit plasma
CMV-DNA, Toxo-DNA test if anti-CMV IgM or anti-Toxoplasma IgM is positive	Before unit release	Segment attached to frozen bag

Question 2

For HPC-CB units collected to enter the Milano Cord Blood Bank inventory – our primary banked product – health donor screening is performed at the delivery suites with a standard donor questionnaire compliant with the national laws, European norms and regulations, and Netcord/FACT standards. Donor selection criteria are focused on risk factors for infectious and genetic transmissible conditions.

Health donor screening for autologous and allogeneic donors of HPC-Marrow, HPC-Apheresis and Lymphocytes-Apheresis units collected in our institution is done in the haematology unit with a standard questionnaire compliant with national laws, Italian Bone Marrow Donor Registry, and JACIE standards. Additional criteria may apply to units received from other institutions, according to local procedures and regulations.

All mothers of HPC-CB donors are screened at banking for anti-HCV, anti-HIV antibodies, HBsAg and Syphilis with standard blood banking serology procedures. Moreover, nucleic acid testing (NAT) for HCV/HIV RNA and HBV DNA is performed at banking. Mothers with positive HCV, HIV, HBV markers are not allowed to donate cord blood for solidaristic allogeneic purposes. Their serology markers negativity is checked 6 months after delivery [3], as prescribed by the current Italian law. Additional testing is performed at unit release, as shown in Table 1, which summarizes HPC-CB testing at the different stages of processing and release and the type of sample used for testing.

HPC-Marrow, HPC-Apheresis and Lymphocytes-Apheresis units screening includes the following: anti-HCV, anti-HIV, anti-HTLV I/II antibodies (the latter is not done for autologous use), HBsAg, anti HBc antibodies and Syphilis. Moreover, nucleic acid testing (NAT) for HCV/HIV RNA and HBV DNA is performed. Sterility for aerobic and anaerobic bacteria and fungi is determined with the Bactec method.

Selection of autologous donors for experimental ATMP protocols is performed by the clinicians incharge of the

patient treatment according to specific inclusion and exclusion criteria of each experimental protocol.

Storage of autologous cryopreserved ATMP from donors with positive HCV markers, who currently participate in an experimental clinical protocol for the treatment of end-stage liver disease, is done in the vapour phase of dedicated liquid nitrogen tanks.

Other infectious markers can be determined upon request of the clinical centre using the product depending on patient characteristics, treatment protocols and national regulations of the requesting centre.

Donor identity checking for HPC-CB, in addition to standard practices of direct donor questioning and unit and samples barcode labelling, is done through comparison of unit HLA typing at banking with confirmatory typing at time of product release. The latter is done on a segment integrally attached to and stored with the bag.

Additional procedures to reduce the risk of unit misidentification include the use of separate areas for unit processing and storage and for administrative activities, the rule to manage one kind of product at any one time and the use of double checks by two operators at critical steps, for example, before detaching two connected bags and when the product is transferred into a new container. All procedures for sample collection requiring the entry of the bag are performed with aseptic techniques under a sterile flow hood.

Question 3

General rules for the characterization of quality of the starting material include the following: (i) inspection and evaluation of the integrity of the package containing the material; (ii) coherence and completeness of material and donor information reported on the material container, the associated samples and the accompanying forms; (iii) evaluation of the compliance between the conditions of material transport to the CTMC including time and temperature with specific limits for each material.

In addition, assays specific for the different materials include the following.

Type I products. (i) HPC-CB to obtain expanded CD34+ cells; (ii) HPC-Marrow and HPC-Apheresis to obtain CD133+ cells; (iii) AT, HPC-Marrow and HPC-CB for the production of autologous and allogeneic MSC: cell counting (internal method); flow cytometry (internal method); viability with Propidium Iodide (P.I.) by flow cytometry; sterility testing (Eu. Ph. 2.6.27). In addition, for expanded MSC, we perform HLA typing (class I), karyotype and telomerase activity.

Type II products. (i) for HPC-CB see Question 2; (ii) autologous HPC-Marrow, HPC-Apheresis: volume, total nucleated cells (TNC), haematocrit, CD34+ cell count and viability, haemopoietic colonies (CFU-GM and CFC) in methylcellulose; in addition, CD3+, CD4+, CD8+, CD19+, NK cell count are determined in allogeneic collections; (iii) Lymphocytes-Apheresis: volume, total nucleated cells (TNC), haematocrit, CD3+, CD4+, CD8+, CD19+, NK cell count and viability.

Question 4

Before releasing any product, we perform a comprehensive check that all the steps during the procedure from collection to release have been performed correctly and are properly documented. In addition, quality control testing of the final cellular therapy product prior to release includes a number of assays as follows.

Type I products. (i) CD133+ cells from HPC-Marrow and from HPC-Apheresis: cell counting (internal method); flow cytometry (internal method); viability with P.I. by flow cytometry; sterility testing (Eu. Ph. 2.6.27) and endotoxins (Eu. Ph. 2.6.14); (ii) expanded CD34+ cells from HPC-CB as in (i) plus mycoplasma (Eu. Ph. 2.6.7); c) MSC: as in (ii), plus karyotype and telomerase activity. Moreover, different potency assays are performed in different protocols (e.g. cytokine secretion and migration assays); in the case of allogeneic use, mixed lymphocyte reaction with recipient's mononuclear cells is performed on multiple MSC lots to screen the best unit for the recipient.

Type II products. (i) HPC-CB see Question 2. Specifically, an integrally attached segment is used to determine nucleated cell count and viability, CFU-GM and CFC. Moreover, we also determine HPC-CB mother's genomic HLA-A,B,DRB1 typing at low resolution to check mendelian inheritance of HLA type; (ii) HPC-Marrow and HPC-Apheresis (vial sample): TNC count and viability (trypan blue), CFU-GM and CFC; Lymphocytes-Apheresis: TNC count and viability.

Question 5a

We have defined the following acceptable ranges/thresholds of the results of release assays.

Type I products.

(i) cell count: for all the products, the range depends on the requested cell dose; (ii) viability (P.I.): ≥80%; (iii) sterility and mycoplasma: for all the products, the results must be 'no growth'; (iv) endotoxins: ≤2·5 IU/ml; (v) flow cytometry: for expanded CD34+ cells from HPC-CB, the absolute value of CD34+ cells depends on the requested cell dose; for CD133+ cells, purity must be ≥80%; for MSC-AT, MSC-Marrow, MSC-CB, the percentage of CD90+/105+/73+/45- cells must be ≥90%; (vi) karyotype: 46;XX or 46;XY (normal karyotype).

Type II products. HPC-CB: see Question 2. Although we do not define a minimum TNC/kg of patient's body weight as

a formal release criteria, we do not recommend to transplant HPC-CB units with a dose below $2{\cdot}5 \times 10^7$ TNC/kg. Acceptable criteria for release of HPC-CB, HPC-Marrow and HPC-Apheresis are any CFC growth and cell viability ≥50%. In the very uncommon cases of 'no colony growth', we inform the clinician and repeat the assay. The final decision to use or not to use the unit is taken by the clinician, who balances the risks and benefits for the recipient. Release criteria for Lymphocytes-Apheresis units are cell viability ≥50%.

Question 5b

Type I products. A specific policy is defined in each clinical trial.

Type II products. See 5a.

Question 6a

Potency assays at release are as follows.

Type I products. For ATMPs, apart from the assays performed using flow cytometry, we do not perform additional potency assay at release.

Type II products. The potency assay for HPC's is the CFC count; see 5a. For Lymphocytes-Apheresis, apart from the immunophenotype, we do not perform any potency assay at release.

Question 6b

See Question 6a.

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The following answers refer only to the Japanese Red Cross Kanto-Koshinetsu Cord Blood Bank, in the Japanese Red Cross Kanto-Koshinetsu Block Blood Centre (formerly in the Japanese Red Cross Tokyo Blood Centre).

Question 1

We deal with cord blood, which is a type II product.

Question 2

Type II product. Questionnaires are filled out by the expectant mothers who volunteer, on their and their family's health history. One of the questionnaires is similar to that given to blood donors, while the other is unique for cord blood donations and inquires about the family history of inheritable diseases. Inheritable diseases in close relatives which can be transferred to the recipient by stem cell transplantation are considered, depending on the mode of the inheritance pattern, such as X-linked recessive, etc. When there have been multiple familial cancers, mother's malignancy or autoimmune disease, the cord blood is not collected. If the delivery and the newborn's condition are without problems, then the cord blood is collected.

Question 3

Type II product. At the start, we require a collected volume of more than 60 ml of cord blood. We start processing the cord blood when the total nucleated cell count (TNC) with an automated counter (XE-2100; Sysmex, Hyogo, Japan) is $11{\cdot}4 \times 10^8$ or more, or if the TNC is between 10 and $11{\cdot}4 \times 10^8$ and the CD34 positive cell count is $4{\cdot}4 \times 10^6$ or more with 7AAD/CD45/CD34 staining using Stem-Kit (stemCXP v2.0/FC500; Beckman Coulter, CA, USA) [1].

The mother's serum is sent to the test section for infectious marker screening, which includes HBs, anti-HBc, anti-HCV, anti-HIV-1/2, anti-HTLV-1, parvovirus B19, syphilis and anti-CMV using chemiluminescence enzyme immuno assay (CLEIA; CL4800, Fujirebio, Tokyo, Japan). Using cord blood plasma separated after it is mixed with 20v/v % HES, the same serological screening tests with CLEIA are performed as well as nucleic amplification tests for HBV, HCV and HIV-1/2. When the mother's anti-CMV IgG/IgM is positive, we perform an anti-CMV IgM EIA (Denka Seiken, Tokyo, Japan) using the cord blood plasma, which will be replaced by a CMV DNA detection test using a sample of the cord blood.