Review Article

Curing Hemoglobinopathies: Challenges and Advances of Conventional and New Gene Therapy Approaches

Irene Motta[1,2], Valentina Ghiaccio[3], Andrea Cosentino[2] and Laura Breda[3].

[1] Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy.
[2] Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy.
[3] Department of Pediatrics, Division of Hematology, Children's Hospital of Philadelphia, Philadelphia, PA, United States.

Competing interests: The authors declare no conflict of Interest.

Abstract. Inherited hemoglobin disorders, including beta-thalassemia (BT) and sickle-cell disease (SCD), are the most common monogenic diseases worldwide, with a global carrier frequency of over 5%.[1] With migration, they are becoming more common worldwide, making their management and care an increasing concern for health care systems.

BT is characterized by an imbalance in the α/β-globin chain ratio, ineffective erythropoiesis, chronic hemolytic anemia, and compensatory hemopoietic expansion.[1] Globally, there are over 25,000 births each year with transfusion-dependent thalassemia (TDT). The currently available treatment for TDT is lifelong transfusions and iron chelation therapy or allogenic bone marrow transplantation as a curative option. SCD affects 300 million people worldwide[2] and severely impacts the quality of life of patients who experience unpredictable, recurrent acute and chronic severe pain, stroke, infections, pulmonary disease, kidney disease, retinopathy, and other complications. While survival has been dramatically extended, quality of life is markedly reduced by disease- and treatment-associated morbidity.

The development of safe, tissue-specific and efficient vectors, and efficient gene-editing technologies has led to the development of several gene therapy trials for BT and SCD. However, the complexity of the approach presents its hurdles. Fundamental factors at play include the requirement for myeloablation on a patient with benign disease, the age of the patient, and the consequent bone marrow microenvironment. A successful path from proof-of-concept studies to commercialization must render gene therapy a sustainable and accessible approach for a large number of patients. Furthermore, the cost of these therapies is a considerable challenge for the health care system. While new promising therapeutic options are emerging,[3,4] and many others are on the pipeline,[5] gene therapy can potentially cure patients. We herein provide an overview of the most recent, likely potentially curative therapies for hemoglobinopathies and a summary of the challenges that these approaches entail.

Correspondence to: Laura Breda. Department of Pediatrics, Division of Hematology, Children's Hospital of Philadelphia, Philadelphia, PA, United States. E-mail: bredal@email.chop.edu

Bone Marrow Transplantation (BMT). One of the most recurring statements in the literature about

hematopoietic stem cell transplantation (HSCT) in thalassemia is "Allogenic bone marrow transplantation is the only available curative treatment for thalassemia major." This approach is rooted in the Italian experience of the early 1980s at the transplant center of Pesaro, where throughout the 1980s and early 1990s, more than 1000 thalassemia major (TM) patients received transplants, and the center reported a 20-year probability of Thalassemia-free survival of 73% in 900 consecutive transplanted patients.[6]

More than 35 years have passed since the first TM patient underwent an allogeneic BM transplantation. Since the pioneering Pesaro experience, the curative role of BM transplantation in TM has been well established, and other European Bone Marrow Transplant (EBMT) centers have routinely performed this therapy.

Indeed, in a retrospective non-interventional study, data analyzed from the EBMT registry database on 1493 consecutive patients with TM transplanted between 1 January 2000 and 31 December 2010. The 2-year overall survival (OS) and event-free survival (EFS) were 88±1% and 81±1%, respectively, after a median observation period of 2 years. OS and EFS were 90%, 81% and 93% (P<0.001), and 82%, 76%, and 85% (P=0.003) in patients who had received bone marrow, peripheral blood, or cord blood (alone or combined), respectively.[7]

The decision to undergo a curative but potentially fatal treatment should be taken with an assessment of transplantation-related mortality (TRM). The Pesaro risk assessment (for a pediatric population) was developed in the early 1990s.[8] This assessment stratifies the outcome of transplantation in three classes on the basis of hepatomegaly, portal fibrosis, and irregular chelation history.[6] According to these risk factors, patients were categorized into three risk classes: Class 1 patients, who had none of these adverse risk factors, class 2 patients, who had one or two adverse risk factors and class 3 patients, who had all three. The thalassemia-free survival (TFS) was respectively 85-90%, 80% and 65-70%, while the transplant-related mortality (TRM) positively increased between classes 1, 2, and 3.[9]

Taking these results into consideration, it is mandatory to offer an HSCT to a young TM patient with a matched sibling donor before the development of iron overload and iron tissue damage. Of note, all the gene therapy clinical trials have as exclusion criteria the presence of a matched sibling donor (MSD).

The rational of bone marrow transplantation in a TM patient is to restore the tissue's capability of producing functional hemoglobin, and that can be achieved even with the coexistence of donor and recipient hematopoietic stem cells. In fact, in approximately 10% of patients, a condition of persistent mixed chimerism in which the donor

hematopoiesis maintain the potential to correct the phenotype of the disease was described. On the other hand, it is vital to minimize the risk of graft-versus-host disease (GVHD), since the immunologic effect of the transplant is unnecessary in a non-malignant disease such as thalassemia.

According to the retrospective EBMT survey concerning the HLA matching of transplant recipients, 1061 (71.1%) HSCT were performed using HLA-identical sibling donors, 127 (8.5%) from another HLA-matched relative, 57 (3.8%) from an unmatched relative and 210 (14.1%) from a matched unrelated donor.[7] This significant disproportion of donor sources is due to the fact that MSD transplants give the safest outcome with a 2-year OS of 91%, compared to 88% of matched family donors and 77% of matched unrelated donors.

In a recent survey, Li et al. analyzed data about BMT in children and young adults with TM comparing the use of alternative donors and HLA-matched related donor in 3 geographic regions: China, India, and the United States. They reported that the 5-year probabilities of OS after HLA-matched relative, HLA-mismatched relative, HLA-matched unrelated, and HLA-mismatched unrelated donor transplants were 89%, 73%, 87% and 83%, respectively. The 5-year probabilities of EFS after HLA-matched relative, HLA-mismatched relative, HLA-matched unrelated, and HLA-mismatched unrelated donor transplants were 86%, 70% and 82%, 78%, respectively.[10] This report is the first demonstrating comparable event-free and overall survival after HLA-matched related and HLA-matched unrelated donor transplantation.

BM transplantation was the only approved curative treatment for TM patient until June 2019, when the first gene therapy product, Zynteglo, was approved by the European Medicine Agency (EMA) for TDT patients who do not entirely lack beta-globin and who are eligible for stem cell transplantation but do not have a matching related donor.[11] Of note, the availability of a suitable donor, together with patient fitness, are the main limitations to the broader use of HSCT. For this reason, there is a need to reduce the toxicity of HSCT, investigate new conditioning strategies, or improve and extend the autologous gene therapy approach also to the most severe cases.

Clinical Trials Based on Gene Therapy and Gene Editing. Currently, a growing number of clinical trials for hemoglobinopathies are investigating the safety and efficacy of gene addition and gene editing based technologies to rescue hemoglobin synthesis in beta-globinopathies. The first and longer-dated include trials indicated for the cure of beta-thalassemia as well as sickle cell disease via beta or gamma-globin gene addition, while the latter is mostly aimed at the cure of SCD via reactivation of fetal hemoglobin. The first

gene addition-based trial that led to cure in a beta0/betaE patient was reported in 2010,[12] and since then, roughly 50 patients with BT and more than 20 patients with SCD have been treated, and the related outcomes have been reported. The data published or communicated on the trials were obtained with four different lentiviral vectors: HPV569, later implemented as Lentiglobin BB305,[13] employed by bluebird bio (formerly Genetix Pharmaceuticals); GLOBE,[14,15] employed by IRCCS San Raffaele, Italy; RVT-1801, employed by CCHMC, Cincinnati, USA (presented at the 22nd ASCGT meeting in 2019), and TNS9.3.55,[16] employed by Memorial Sloan Kettering Cancer Center NY, USA. This trial (NCT01639690) was subsequently halted, and only one patient (out of 4 treated) had a significant decrease in transfusion requirements. An additional trial has been initiated by UCLA, using the AS3-FB vector,[17] but no data related to patients' outcome has been released so far. **Table 1** lists the trial identifiers as well as the specifics of vectors and regimens employed in each trial. The significant challenges for these trials relate to two major determinants intrinsic to hemoglobinopathies: the severity of the hemoglobin defect in BT (i.e., beta0/0 genotype being the most challenging to correct) as well as the disease progression for both BT and SCD. Initial trials, focused on adult patients with a beta+/+ or beta+/0 BT, had a fair degree of success, where patients became mostly transfusion independent even with suboptimal level (less than two copies per genome) of lentiviral integration or vector copy number (VCN) (NCT01745120 and NCT02151526). This level of integration proved to be unsuccessful at achieving transfusion independence for most of the adult patients affected by BT due to a beta0/0

genotype.[18] Therefore, subsequent trials have aimed at optimizing transduction protocols to obtain higher VCN (roughly 3-4 copies) in the drug product (DP), which indicates a patient hematopoietic stem cell (HSC) transduced with the lentiglobin vector, and maximizing transgenic chimerism, which indicates the proportion of HSC successfully transduced and engrafted. Patients with beta0/0 BT and SCD have benefited from the new transduction regimens, achieving hemoglobin (Hb) levels that do not require transfusion and therefore are considered curative (NCT02906202 and groups B and C of NTC02140554, see **Table 1** for details). In light of these results, EMA recently approved bluebird bio's Zintheglo, the first drug for gene therapy in TDT, in Europe. We learned that disease progression, hence the age of a patient, can greatly affect the outcome of gene therapy. Actually, the trial conducted in Italy (NCT02453477), using GLOBE, showed that the same protocol produced the best outcome in the youngest cohort (pediatric), while it was progressively less successful for the middle and oldest ones.[19] More data on the pediatric population will be necessary to better elucidate the correlation between curative outcome and age.

Additionally, in the first seven subjects with SCD (NTC02140554-group A), for whom BM-derived HSC were utilized, the median expression of vector-derived hemoglobin was only 0.4 g/dL (range 0.1 to 1 g/dL). Both cases suggest that the BM environment in hemoglobinopathies is not fully understood, and its physiology might be progressively altered by disease progression. Better results were achieved by the procurement of HSC from peripheral blood via apheresis, using improved mobilization practices, as discussed in the section that follows.

Table 1

Trial identifier	Hemoglobinopathy/ number of patients	Myeloablative regimen	Mobilization protocol	CD34+ infused (million/kg)	Vector	VCN in DP (Median)	Sponsor/ Center
NCT02186418	SCD (2)	IV Melphalan	BM harvest/ Plerixafor	1 and 6.9	RVT1801	0.3	Aruvant/ CCHMC
NCT02453477	BT (9, of which 6 pediatric)	Treosulfan and Thiotepa	G-CSF and Plerixafor	19.5 (I.O. infusion)	GLOBE	0.93	San Raffaele Telethon Institute for Gene Therapy (SR-TIGET) Italy
NCT01745120 (HGB-204)	BT (18 as of 4/2018)	Busulfan	G-CSF and Plerixafor	8.1	BB305	0.7	bluebird bio (multicenter, 6 sites)
NCT02151526 (HGB-205)	BT and SCD (4 as of 4/2018)	Busulfan (adjusted based on daily PK monitoring)	G-CSF and Plerixafor (after 3 months of enhanced transfusion)	10.5	BB305	1.3	bluebird bio (Necker Children's Hospital in Paris)
NCT02140554 (HGB-206) 3 groups: A, B, C	SCD (7, A); (2, B); and (9, C)	Busulfan	BM harvest (A, B)/ Plerixafor (C)	2.1 (A) 2.7 (B) 6.5 (C)	BB305	0.6 (A) 3.1 (B) 3.8 (C)	bluebird bio (USA)
NCT02906202 (HGB 207)	Non beta0/0 BT including pediatric (16)	Busulfan	G-CSF and Plerixafor	NA	BB305	3.1	bluebird bio (international, multicenter)
NCT03207009 (HGB 212)	beta0/0 BT (3)	Busulfan	G-CSF and Plerixafor	NA	BB305	NA	bluebird bio (international, multicenter)

The first clinical data based on a gene-editing approach were recently communicated for the clinical trial NCT03282656 for severe SCD (ASH 2018), which has been conducted at the Boston Children's Hospital, MA, by bluebird bio. As for most of the gene addition studies above mentioned, this pilot trial uses Plerixafor to mobilize patients' HSC and a busulfan regimen for conditioning. The first three patients presented at ASH 2018 were infused with a drug product (HSC count: from 3.3 to 6.7 X 10^6/kg weight) that had a VCN between 3.25 and 5. The drug product was transduced (over 90% rate) with BCH_BB-LCRshRNA (miR),[13] a lentiviral vector that carries a short hairpin RNA targeting BCL11A, a known repressor of gamma-globin expression.[20] The first patient treated and followed for six months presented a 24% increase in HbF, a high number of F expressing cells in circulation (72%), a considerable reduction in reticulocytosis as well early mitigation of some of the cellular phenotype of SCD. Enrollment for this trial is still active and will involve a total of 7 patients.

Since naturally-occurring deletional mutations of the binding site of BCL11A in the gamma-globin promoter have been found to be associated with cases of high persistence of fetal hemoglobin (HPFH), this very same deletion has been the target of the newest gene-editing approaches for SCD. Two trials, enrolling patients in more than one site within the US, NCT03745287 and NCT03653247, are been conducted by CRISPR Therapeutics and Bioverativ (Sanofi), respectively.

Although based on the same target, the CRISPR therapeutics approach relies on the use of CRISPR/CAS9 to edit patients' HSC, while Sanofi's approach relies on the use of a zinc-finger nuclease. An additional trial by Sangamo, NCT03432364, also uses a zinc-finger nuclease approach to target the enhancer of BCL11A, although this treatment is indicated for BT. Preliminary results, presented by Sangamo at ASCGT 2019, showed successful gene editing of peripheral white blood cells collected from the first treated patient, a beta0/0 BT case, an increase of fetal Hb levels, and stable total Hb values around 9g/dL at 50 days from the infusion. Longer follow-up periods for all these most recent studies are needed to further elucidate the extent of the success of the gene-editing technology for hemoglobinopathies.

Conditioning in Clinical Trials and New Preclinical Development. The conditioning regimen for hemoglobinopathies does not include drugs with specific antitumor effects, given the non-malignant nature of these diseases.[21] Nevertheless, to achieve stable engraftment, patients receive chemotherapy that aims to make space, eliminating an extremely proliferating marrow with erythroid hyperplasia and favor engraftment of HSC.[22,23]

Data from reduced-intensity allogeneic transplantation in limited clinical series show that the achievement of stable donor engraftment is difficult in immunocompetent patients with hemoglobinopathies.[24,25] Therefore, patients with BT and SCD need more intensive myeloablative conditioning regimens in order to reduce the risk of graft failure.[23] Myeloablative conditioning is associated with short- and long-term toxicity in hematopoietic and non-hematopoietic tissues, including cancer and infertility and gonadal failure, particularly among females.[26] The ideal regimen should combine an efficient depletion of HSC and minimized toxicity.

Non-myeloablative reduced-intensity conditioning, based on busulfan at a total dose of 8 mg/kg, has been used in the gene addition trial (NCT01639690) at Memorial Sloan Kettering Cancer Center[27] and resulted in insufficient engraftment of gene-marked cells and minimal clinical benefit.[28]

In two phase 1/2 GT trials (NCT01745120 and NCT02151526) for TDT with LentiGlobin BB305 vector, myeloablative conditioning with busulfan as a single agent was used. A dose adjustment was performed to achieve appropriately targeted drug exposure. The average daily plasma busulfan area-under-the-curve values ranged from 3029 to 4714 μM per minute in HGB-204 (estimated values) and from 4670 to 5212 μM per minute in HGB-205 (actual values). Among the 22 treated patients, 9 serious adverse events (SAE) were reported, including two episodes of veno-occlusive liver disease attributed to busulfan conditioning.[18] Busulfan was also used for SCD patients in several trials (NCT02151526, NCT03282656, NCT02247843) with mixed results.[29]

The phase 1/2 GT trial for TDT at IRCCS San Raffaele in Milan, used myeloablative conditioning with treosulfan–thiotepa, given their reduced extramedullary toxicity compared to busulfan. Chemotherapy-related toxicity was mild, and 5 SAE of infectious nature were reported and resolved. Neither veno-occlusive disease nor hepatic toxicity was observed.[19] Most recently, preliminary results from a reduced-intensity conditioning (RIC) phase 1/2 pilot study on gene transfer for SCD were presented.[30] For this trial, a single dose of melphalan is used as the conditioning regimen, while patients' HSC are corrected using a γ-globin based lentiviral vector (RVT1801; NCT02186418). Results from this trial showed excellent safety, feasibility, with minimal post-transplant toxicity, rapid count recovery, and sustained stable genetically modified cells in peripheral blood and bone marrow.

Although autologous HSCT using genetically corrected cells would avoid the risk of graft-versus-host disease (GVHD), the genotoxicity of conditioning remains a substantial barrier to the development of this

approach in non-malignant disorders. The toxicities of conditioning lessen the willingness of patients and healthcare providers to consider this therapy. Novel strategies that aim to reduce regimen-related toxicity, remaining on the other hand sufficiently myeloablative, are based on the use of antibodies that specifically target HSC and other hematopoietic cells in the bone marrow niche, sparing non-hematopoietic cells.[31,32] If proof of safety and efficacy stands, these new regimens will satisfy the need for reduction of morbidity and mortality related to the current conditioning regimens, mainly based on busulfan administration.

In mice, immunotoxin CD45–saporin (SAP) allows high-level engraftment and multi-lineage repopulation of transplanted HSC without the need for chemotherapy or irradiation and enables efficient engraftment of donor cells and full correction of a sickle-cell anemia model.[31]

The depletion of HSC can also be achieved using a c-kit (CD117) antibody, as its receptor is highly expressed on these cells. Binding of CD117 ligand is essential for hematopoiesis and self-renewal. The internalization of the receptor by antibody binding causes HSC failure. A CD117 antibody molecule conjugated with a non-genotoxic small toxin molecule can deplete more than 98% of human HSC in NSG mice, leaving intact T and B cells.[33] Instead, the unconjugated antibody presents a much-reduced effect. The velocity of the internalization of the receptor and selectivity of the CD117 molecule makes this method an excellent candidate for gene-corrected BMT for hematopoietic conditions, like BT and SCD, preventing the systemic toxicity typical of irradiation and chemotherapy regimens.

A clinical study, carried out at Stanford (NCT02963064), utilizes this very strategy to condition patients with severe combined immunodeficiency disease prior to HSCT. This study aims at demonstrating the safety and efficacy of AMG191, a CD117 conjugated antibody given intravenously in one dose, followed by infusion of donor CD34+CD90+ HSC. This purified fraction of HSC that can be isolated by flow cytometry has been shown to possess the stem-like quality necessary to repopulate the BM in long term studies in nonhuman primates.[34] Patients that undergo this procedure receive 1X106 CD34+CD90+ cells/kg, a much lesser dose than that one used when the CD34 marker alone is selected. The antibody clearance can be monitored via PK study to establish the best time for the infusion of donor cells without compromising their chance to engraft the BM niche. This protocol includes subjects with a poor graft from previous HSCT, indicating that this could be performed in patients whose prior BMT has not succeeded with chemotherapy agents. If successfully and safely applied to patients with hemoglobinopathies, this technology could positively impact not only patients' health, by

eliminating the myeloablation related toxicity, but also limit the cost of gene therapy, by limiting the number of cells that needs to be corrected.

CD34+ Hematopoietic Progenitor Cells Mobilization. Peripheral stem cells are commonly used for transplantation and have replaced bone marrow as a source of HSC in most transplants, especially in the autologous setting.[35] Granulocyte-colony stimulating factor (G-CSF) is the standard agent to mobilize hematopoietic stem and progenitor cells (HSPC) for transplantation. However, peculiar features of hemoglobinopathies, namely splenomegaly and thrombophilia state, may represent risk factors for adverse events. G-CSF-related enlargements of the spleen, rarely resulting in splenic rupture,[36-38] and thrombotic events[39] have been reported.

Moreover, in adult thalassemia patients iron-overload and consequent oxidative stress, the suppressive effect of long-term transfusions and chelation on the stem cell compartment, and the "aged" stem cells, could compromise the safety and success of HSC procurement.[40] Also, in SCD, the use of G-CSF has been associated with severe and life-threatening vaso-occlusive complications.[41,42] G-CSF induces an increase in white blood cells count, neutrophils, endothelial cells, platelets, and coagulation activation, all mechanisms that contribute to the vaso-occlusive crisis.

Among the other compounds available, Plerixafor represents a good alternative. Plerixafor is a bicyclam molecule that selectively and reversibly prevents the binding of stromal-derived factor-1 (SDF-1) to chemokine CXC-receptor-4 (CXCR4) on HSPC, inducing their mobilization.[43] Plerixafor has proved to be safe and effective in mobilizing HSC in thalassemia patients.[18,19,44]

It has been shown that Plerixafor and G-CSF mobilize different primitive HSC populations, either in thalassemia patients with thalassemia or healthy donors. Plerixafor-mobilized cells have a "stemness" signature compared to G-CSF mobilized cells, and the combined use of the two agents attenuates this "stemness." Furthermore, Plerixafor-mobilized HSC possess the highest ability to home to hematopoietic niches and engraft in immunodeficient mice, and their global gene expression signature highlights their superior *in vivo* reconstitution activity.[45]

Currently, several phase 1 and 2 trials are evaluating the safety and efficacy in collecting a sufficient number of HSC with Plerixafor in SCD patients (NCT02989701, NCT03226691, NCT02193191, NCT02212535, NCT02140554). Lagresle-Peyrou's group published the results of a French trial; no adverse events were observed administrating Plerixafor in a single-dose of 240 mcg/kg in three patients who had discontinued hydroxyurea (HU). Moreover, with single

apheresis, they were able to collect a high number of HSC.[46] Interim results from a Memorial Sloan Kettering Cancer Center trial with Plerixafor at escalating dose reported data on 15 patients. Ten were on HU and one on chronic transfusion regimen. Two serious adverse events (pain crisis) have been observed at 80 and 240 mcg/kg of Plerixafor, and only 33-50% of patients, according to different doses, reached the target yield of HSC.[47] Most recent data on group C from the HGB-206 study (NCT02140554) show that mobilization was effective in SCD patients with Plerixafor at the dose of 240 mcg/kg. No life-threatening VOCs after Plerixafor mobilization have been reported.[48]

Three main considerations can be drawn from the studies on conditioning conducted thus far. One relates to HU administration prior to the mobilization. HU reduces the amount of circulating CD34+,[49] is associated with myelosuppression, and did not show any beneficial effect in thalassemia patients.[40,50] In the French trial, patients discontinued HU 3 months before the mobilization. However, in the New York trial, no association was observed between HU and the peak of HSC. The second concerns the maintenance of HbS levels>30% in order to prevent the vaso-occlusive crisis. In the French trial (NTC02212535), during the three months before the mobilization, patients underwent a transfusion or erythro-exchange program.[46] The third is the timing of apheresis. The peak of circulating HSC in SCD patients have been observed at 3-6 hours, earlier compared to healthy donors (6-12 hours)[51] in whom apheresis is recommended to start at 11 hours after Plerixafor administration.

From Clinical Trials to Drug Commercialization, the Challenges of Pivotal GT Studies. Because of their monogenetic etiology, both BT and SCD are attractive targets for curative approaches as gene addition and gene editing.

Gene addition strategies have significantly improved over the past ten years and have provided the most successful results thus far. Although these approaches may seem straightforward given the single gene defect and defined cell target, there are still several hurdles that can impact their success, as previously reported.[52]

One of the most relevant challenges is to guarantee a level of functional beta-globin protein expression that can rescue the complete lack of endogenous adult hemoglobin protein, like that seen in patients with beta0/0 BT. The constructs employed in clinical trials utilize large genomic regulatory elements that are essential to express high and tissue-specific expression of the gene of interest, and they are engineered to maximize their safety. The need for such large constructs on the other side can affect the viral assembly and entry, impacting the yield of viral particles that can be functionally assembled during manufacturing, and limiting the number of particles that can effectively enter the target HSC, respectively.

Viral vector-based gene therapy has become commonplace in both the laboratory and the clinic, and it is rapidly evolving towards becoming a curative treatment option. Currently, several products are under clinical development, and the European Medicine Agency recently approved the first lentiviral product (Zynteglo, based on BB305) for gene addition based therapy for BT. The transition from clinical development to commercialization adds another layer of complexity to the picture. Thus, the most pressing concern becomes no longer to make safe, functional lentiviral vectors solely, but to also refine current processes in order to achieve both yield and quality while reducing costs, processing times, and risks to support research, development, and commercialization.

lentivirus-based gene and cell therapy products were estimated in 2017 to cost in the range of U.S. $500,000–1,000,000 per patient,[53] a third of which pertains to the lentivirus manufacturing process itself.

Access to gene therapy to a large number of patients with a hemoglobinopathy is highly dependent on a reliable high-scale lentiviral production. The implementation of scalable vector production protocols is indispensable to fulfill not only the demand of academic and hospital institutions, but also of the industry, which is incentivized to satisfy the upsurge of requests for safe and efficient products.

Lentiviral Vectors Manufacturing. Lentiviral vector manufacturing technologies are based primarily on fully transient, adherent processes. These 2-dimension methods are tedious, time-consuming, and difficult to scale up, with carry-associated issues of process handling, results inconsistency, and high costs. Small-scale productions for R&D purposes are performed transfecting adherent 293T cells grown in plasticware (Petri dishes, T-flasks, Cell Factories, Cell Stacks, or HYPERFlask) in the presence of either PEI or Calcium Phosphate (CaPho).[54,55]

Conversely, large-scale vector manufacturing generally requires a direct scale-up from small-scale systems to increase volumes and titers, and reduce variability. This can be accomplished by using alternative culture devices (e.g., roller bottles, multi tray cell factories, or microcarriers in stirred tanks) that provide extended anchorage surface for adherent 293T cells. Valkama AJ et al.[56] demonstrated how parameters, such as cell density, pH levels, and transfection methods, play a delicate role for large-scale lentiviral production. In their study, they use PALL iCELLis, a compact fixed-bed bioreactor with an integrated perfusion system, comparing yields achieved with either CaPho precipitation or PEI

transfection methods, within a range of pH and under glucose and lactate concentration monitoring. In both transfection methods, they confirmed the critical role of cell density and pH in the efficiency of productivity,[57] showing higher production at high cell-density and mildly acidic pH, with vp cm−2 (vp= viral particle) between 1.31E+08 and 4.85E+08 using iCELLis Nano fixed-bed bioreactors.

To overcome the need for traditional large-scale technologies in lentiviral production, some investigators have engineered novel suspension-adapted cell lines.[58] Although there are no established and standardized methods based on this suspension approach with a Good Manufacturing Practices (GMP) grade approval, several studies are ongoing, aiming to fill the demand for a robust supply chain for GMP materials.

Suspension-adapted cell lines can easily grow at high cell densities in a packed-bed bioreactor, stirred tanks, or Erlenmeyer flasks agitated on orbital shakers, which require minimum handling or supplementary laboratory equipment.[55] Several cell lines (293T, 293FT, and 293SF-3F6) have already been used for the purpose, through a modification that allows growth in suspension in chemically defined media (Freestyle 293 and F17, Invitrogen; HyQSFM4TransFx293).[58,59] Suspension-adapted cell lines grow promptly rendering, thus their maintenance and propagation much easier than that of adherently growing cells. Another advantage of the use of this system in clinical manufacturing is the lack of requirement for bovine serum and animal origin components in the culture media, which decreases the risk of contamination by adventitious agents.

The preferential vessel for large-scale lentiviral production using suspension-adapted cells is a bioreactor. In this type of system, DNA precipitation using calcium phosphate is expected to be less effective for transfecting suspension cells because of continuous culture stirring. Other transfection agents like cationic polymers are used instead. Using linear or branched 25-kDa PEI, Durocher et al. achieved the highest transfection efficiency in 293T and 293-EBNA1 cells, leading to 75% of transfected cells using a green fluorescent protein (GFP) reporter plasmid.[60] McCarron et colleagues were able to reach yields of up to 10^9 TU/mL (TU= transducing units) using a packed-bed bioreactor system, suggesting a more significant potential on the basis that the total packed-bed surface area is 18m^2, which is equivalent to approximately 28 standard 10-layer cell factories.[61]

The viral supernatant is collected from suspension-adapted cells in the bioreactor by anion exchange chromatography. The eluted material can then be concentrated using tangential flow filtration (TFF), and the vector may then be diafiltered into its final formulation buffer.[62]

More recently, developed protocols for lentiviral manufacturing employ hollow fiber bioreactor, suspension culture processes, and the implementation of stable producer cell lines. The use of stable producer cell lines allows to further scale-up production, having the advantage to remove any undesirable process-derived contaminant, such as plasmid or host cell DNA or host cell proteins.[63] The biomedical industry is the driving force to make the use of all these technologies accessible for large-scale platforms in terms of manufacturing, costs, and GMP-compliance. Some of these innovative technologies were presented last May at the 22nd ASCGT meeting. Two independent companies showed the generation of a suspension-adapted cell line GMP-compliant for lentiviral packaging using an inducible Tet system,[64] and production of lentiviruses up to 200L in PALL iCELLis500, reaching 80.1% transduction efficiency in T-cells using a Multiplicity of Infection MOI=4,[65] respectively.

HCS Transduction and Handling. Downstream the lentiviral production *per se*, the manufacturing of the drug product (DP), which consists of patients' HSC corrected with a lentivirus, represents another benchmark for innovative and unconventional protocols that aim to make the process more efficient and scalable. Efficient HSC transduction requires highly concentrated and purified lentiviral particles and, in diseases like beta0/0 BT, in which a higher number of integrations are necessary to achieve curative outcomes, integration of large transgenic cassette can be particularly challenging. Resistance to infection has been attributed to the quiescent (G0) phase and the innate immune defenses against viral transduction.[66] Given the need to achieve optimal integration in the DP that guarantees curative effects without compromising genome safety, several experimental transduction protocols, and agents to enhance/modulate lentiviral gene transfer yield have been developed. The necessity to make the manufacturing of the DP as consistent and reproducible as possible has been driving force for these technologies.

Transduction efficiency can be improved, even at low MOIs, using small molecules like cationic-liposomes, polycations, or small peptides as adjuvant-enhancer of transduction. For over a decade, cationic polymers such as hexadimethrine bromide (Polybrene) have been employed in laboratory-based protocols to neutralize the nonspecific electrostatic interactions between virus and target cell.[67,68] Due to its negative effect on cell growth and viability, polybrene is not indicated for clinical use. Therefore, new molecules have been engineered to promote vector-cell contacts similarly. Balancing safety and transduction efficiency has shown to be pivotal in the process of providing new adjuvants. More recently, Vectofusin-1,[69]

protamine sulfate,[70] and fibronectin fragments[71] have shown to increase transduction efficiency by facilitating adhesion and fusion between the viral cap side and the cell membrane.

LentiBOOST™ reagent, a large nonionic amphiphilic molecule (poloxamer), has been extensively used as a transduction enhancer, with greater activity and better safety profile than similar known nonionic molecules.[72] Human peripheral blood-derived CD34+ HSC transduced with standard MOI of 10, and LentiBOOST™ show increased gene expression and VCN proportional to adjuvant concentration with unaltered viability. The use of LentiBOOST™ is compatible with spinoculation protocols, and its use in combination with protamine sulfate shows an addictive effect.[73] Moreover, the use of RetroNectin and LentiBOOST™ in human HSC transduction concurrently decrease electrostatic interactions and promote either adhesion/fusion and integration.[74]

Prostaglandin E_2 (PGE2) is a molecule that enhances transduction prior to nuclear entry and integration, possibly by interfering in the endocytosis-dependent pathways.[75] While initially intended as an anti-apoptotic agent in experiments on HSC, prestimulation with PGE2 surprisingly showed a ~1.5 fold increase of VCN, both *in vivo* after xenotransplantation.[76] Additionally, the combination of PGE2 and poloxamer synperonic F108 had a synergistic effect, increasing gene transfer in primitive HSC by ~10 fold in CD34+ HSPC with a globin-based lentivirus, which is highly desirable when high VCN is needed.

Viral integration can also be facilitated by molecules that modulate intracellular processes, like Rapamacyn (Rapa). By inducing autophagy through allosteric inhibition, Rapa enhances post-binding endocytic events, increasing lentiviral entry, reverse transcription, and genomic integration.[77] In mouse and human HSC, transduction with 5 to 20 μg/mL of Rapa significantly increase VCN, without loss of viability or engraftment (after transplantation). Furthermore, the role of Rapa on transcriptional and translational events could benefit the integration of lentiviruses by altering chromosomal accessibility.[78] Cyclosporine H has recently been shown to efficiently enhance gene transfer bypassing the innate immune block that can interfere with efficient gene transfer in HSC. Unlike Cyclosporine A, which inhibits the host factor CypA, Cyclosporine H inhibits the interferon-induced transmembrane protein 3 (IFITM3) whose high expression potently restricts VSV-G pseudotyped LV entry.[79]

A different approach, based on optimization of cell density, was used in a recent study, which suggested that cell-to-cell contact in a high-density (4e6/mL) cell culture may mimic bone marrow environment,

allowing more efficient transduction. Ultimately, the combination of PGE2 and Poloxamer at high-density resulted in an even higher lentiviral transduction.[80]

The handling of patients' HSC is just as sensitive, if not more, as the process of making GMP-grade lentiviral particles. Several instruments and trained personnel are needed to carry out the operations that involve HSC apheresis, CD34+ selection, transduction, and cryopreservation in conventional open environments. Even though individual automated instruments are currently used for each step of the process, the entire procedure could benefit from a fully automated closed-system that cuts procedure time, the risk associated with human error, and costs, which could have more drastic implications once the drug product reaches commercialization.

A semi-automated immunogenetic selection system, the CliniMACS Plus by Miltenyi Biotec, is now approved in many countries for autologous CD34+ selection from mobilized apheresis products. The instrument uses a disposable tubing set that allows all significant steps of processing to happen in a closed system, thus minimizing the risk of contamination, a critical factor in cGMP compliance. This method has proved to be successful at reproducing consistent yields among many centers.[81] An advanced version of it, the CliniMACS Prodigy, combines in one instrument the immunoseparation component with a chamber for further cell manipulation in a closed and fully automated system. The chamber has a built-in centrifuge and is temperature controllable from 4C – 37C, making it also an incubator. Briefly, apheresis products undergo to immunomagnetic selection with nanobead-conjugated anti CD34 antibodies and buffer purification in a connected-sterile system with minimum need of operator attendance.[82] This system is currently used in clinical studies for CAR-T manufacturing and allows after CD34 selection to perform lentiviral transduction, further purification and expansion (up to 12 days) within the same closed system. The end of the process sees the transduced target cells resuspended into the formulation buffer and potentially ready to be infused into the patient as drug product.[83]

Summary and Conclusions. At the moment, the only available curative treatment for patients with BT and SCD is allogeneic HSCT. HLA matched sibling donor HSCT is now a well-proven therapy for SCD and BT; however, most patients (>75%) do not have access to this option due to lack of an available matched sibling. Alternative donor allogeneic HSCT using unrelated and haploidentical related donors that would be required for the cure in most patients with SCD and BTM remain limited by high rates of transplant-related mortality, GVHD, and graft rejection. Given the severity of BT and SCD and the shortcomings of

current curative treatment options, BT and SCD remain challenging diseases with a significant unmet medical need, which is even more aggravated in countries with poor access to health care. There is a clear need for potentially curative therapies that can circumvent or eliminate the deficiencies of current options, and significantly improve associated morbidity and mortality.

Extensive progress has been made on gene therapy since its pioneering, making it a nearly available option for patients. Results from clinical trials of the last decade indicate that the use of lentiviral vectors can cure patients affected by hemoglobinopathies. At the moment, in order to correct the phenotype, many patients require high level of integrations per genome (~4) in a pancellular fashion. New challenges are represented by the need to transfer this technology from small to large-scale volumes, to launch its commercialization. As the medical field progresses with new and promising pharmacological therapeutic options, so does the urgency to find better regimens for mobilization and conditioning of patients for patients that opt to undergo gene therapy.

Most importantly, the benefit of the new gene therapy approaches that are potentially curative would be reinforced if made accessible to a large number of patients and affordable for the health care system.

References:

1. Taher AT, Weatherall DJ, Cappellini MD. Thalassaemia. Lancet. 2018;391(10116):155-167.
https://doi.org/10.1016/S0140-6736(17)31822-6
2. Piel FB, Steinberg MH, Rees DC. Sickle Cell Disease. N Engl J Med. 2017;376(16):1561-1573.
https://doi.org/10.1056/NEJMra1510865
PMid:28423290
3. Piga A, Perrotta S, Gamberini MR, Voskaridou E, Melpignano A, Filosa A, Caruso V, Pietrangelo A, Longo F, Tartaglione I, Borgna-Pignatti C, Zhang X, Laadem A, Sherman ML, Attie KM. Luspatercept improves hemoglobin levels and blood transfusion requirements in a study of patients with beta-thalassemia. Blood. 2019;133(12):1279-1289.
https://doi.org/10.1182/blood-2018-10-879247
PMid:30617198 PMCid:PMC6440118
4. Cappellini MD, Porter J, Origa R, Forni GL, Voskaridou E, Galacteros F, Taher AT, Arlet JB, Ribeil JA, Garbowski M, Graziadei G, Brouzes C, Semeraro M, Laadem A, Miteva D, Zou J, Sung V, Zinger T, Attie KM, Hermine O. Sotatercept, a novel transforming growth factor beta ligand trap, improves anemia in beta-thalassemia: a phase II, open-label, dose-finding study. Haematologica. 2019;104(3):477-484.
https://doi.org/10.3324/haematol.2018.198887
PMid:30337358 PMCid:PMC6395345
5. Cappellini MD, Motta I. New therapeutic targets in transfusion-dependent and -independent thalassemia. Hematology Am Soc Hematol Educ Program. 2017;2017(1):278-283.
https://doi.org/10.1182/asheducation-2017.1.278
PMid:29222267 PMCid:PMC6142569
6. Lucarelli G, Galimberti M, Polchi P, Angelucci E, Baronciani D, Giardini C, Politi P, Durazzi SM, Muretto P, Albertini F. Bone marrow transplantation in patients with thalassemia. N Engl J Med. 1990;322(7):417-421.
https://doi.org/10.1056/NEJM199002153220701
PMid:2300104
7. Baronciani D, Angelucci E, Potschger U, Gaziev J, Yesilipek A, Zecca M, Orofino MG, Giardini C, Al-Ahmari A, Marktel S, de la Fuente J, Ghavamzadeh A, Hussein AA, Targhetta C, Pilo F, Locatelli F, Dini G, Bader P, Peters C. Hemopoietic stem cell transplantation in thalassemia: a report from the European Society for Blood and Bone Marrow Transplantation Hemoglobinopathy Registry, 2000-2010. Bone Marrow Transplant. 2016;51(4):536-541.
https://doi.org/10.1038/bmt.2015.293
PMid:26752139
8. Angelucci E, Pilo F, Coates TD. Transplantation in thalassemia: Revisiting the Pesaro risk factors 25 years later. Am J Hematol. 2017;92(5):411-413.
https://doi.org/10.1002/ajh.24674
PMid:28181283
9. Lucarelli G, Isgro A, Sodani P, Gaziev J. Hematopoietic stem cell transplantation in thalassemia and sickle cell anemia. Cold Spring Harb Perspect Med. 2012;2(5):a011825.
https://doi.org/10.1101/cshperspect.a011825
PMid:22553502 PMCid:PMC3331690
10. Li C, Mathews V, Kim S, George B, Hebert K, Jiang H, Li C, Zhu Y, Keesler DA, Boelens JJ, Dvorak CC, Agarwal R, Auletta JJ, Goyal RK, Hanna R, Kasow K, Shenoy S, Smith AR, Walters MC, Eapen M. Related and unrelated donor transplantation for beta-thalassemia major: results of an international survey. Blood Adv. 2019;3(17):2562-2570.
https://doi.org/10.1182/bloodadvances.2019000291
PMid:31471325 PMCid:PMC6737407
11. Zynteglo. European Medicine Agency (EMA). https://www.ema.europa.eu/en/medicines/human/EPAR/zynteglo. Accessed October 20, 2019.
12. Cavazzana-Calvo M, Payen E, Negre O, Wang G, Hehir K, Fusil F, Down J, Denaro M, Brady T, Westerman K, Cavallesco R, Gillet-Legrand B, Caccavelli L, Sgarra R, Maouche-Chretien L, Bernaudin F, Girot R, Dorazio R, Mulder GJ, Polack A, Bank A, Soulier J, Larghero J, Kabbara N, Dalle B, Gourmel B, Socie G, Chretien S, Cartier N, Aubourg P, Fischer A, Cornetta K, Galacteros F, Beuzard Y, Gluckman E, Bushman F, Hacein-Bey-Abina S, Leboulch P. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. Nature. 2010;467(7313):318-322.
https://doi.org/10.1038/nature09328
PMid:20844535 PMCid:PMC3355472
13. Negre O, Bartholomae C, Beuzard Y, Cavazzana M, Christiansen L, Courne C, Deichmann A, Denaro M, de Dreuzy E, Finer M, Fronza R, Gillet-Legrand B, Joubert C, Kutner R, Leboulch P, Maouche L, Paulard A, Pierciey FJ, Rothe M, Ryu B, Schmidt M, von Kalle C, Payen E, Veres G. Preclinical evaluation of efficacy and safety of an improved lentiviral vector for the treatment of beta-thalassemia and sickle cell disease. Curr Gene Ther. 2015;15(1):64-81.
https://doi.org/10.2174/1566523214666141127095336
PMid:25429463 PMCid:PMC4440358
14. Miccio A, Cesari R, Lotti F, Rossi C, Sanvito F, Ponzoni M, Routledge SJ, Chow CM, Antoniou MN, Ferrari G. In vivo selection of genetically modified erythroblastic progenitors leads to long-term correction of beta-thalassemia. Proc Natl Acad Sci U S A. 2008;105(30):10547-10552.
https://doi.org/10.1073/pnas.0711666105
PMid:18650378 PMCid:PMC2492493
15. Lidonnici MR, Paleari Y, Tiboni F, Mandelli G, Rossi C, Vezzoli M, Aprile A, Lederer CW, Ambrosi A, Chanut F, Sanvito F, Calabria A, Poletti V, Mavilio F, Montini E, Naldini L, Cristofori P, Ferrari G. Multiple Integrated Non-clinical Studies Predict the Safety of Lentivirus-Mediated Gene Therapy for beta-Thalassemia. Mol Ther Methods Clin Dev. 2018;11:9-28.
https://doi.org/10.1016/j.omtm.2018.09.001
PMid:30320151 PMCid:PMC6178212
16. Boulad F, Wang X, Qu J, Taylor C, Ferro L, Karponi G, Bartido S, Giardina P, Heller G, Prockop SE, Maggio A, Sadelain M, Riviere I. Safe mobilization of CD34+ cells in adults with beta-thalassemia and validation of effective globin gene transfer for clinical investigation. Blood. 2014;123(10):1483-1486.
https://doi.org/10.1182/blood-2013-06-507178
PMid:24429337 PMCid:PMC3945860
17. Romero Z, Urbinati F, Geiger S, Cooper AR, Wherley J, Kaufman ML, Hollis RP, de Assin RR, Senadheera S, Sahagian A, Jin X, Gellis A, Wang X, Gjertson D, Deoliveira S, Kempert P, Shupien S, Abdel-Azim H, Walters MC, Meiselman HJ, Wenby RB, Gruber T, Marder V, Coates TD, Kohn DB. beta-globin gene transfer to human bone marrow for sickle cell disease. J Clin Invest. 2013.
https://doi.org/10.1172/JCI67930

PMid:23863630 PMCid:PMC4011030

18. Thompson AA, Walters MC, Kwiatkowski J, Rasko JEJ, Ribeil JA, Hongeng S, Magrin E, Schiller GJ, Payen E, Semeraro M, Moshous D, Lefrere F, Puy H, Bourget P, Magnani A, Caccavelli L, Diana JS, Suarez F, Monpoux F, Brousse V, Poirot C, Brouzes C, Meritet JF, Pondarre C, Beuzard Y, Chretien S, Lefebvre T, Teachey DT, Anurathapan U, Ho PJ, von Kalle C, Kletzel M, Vichinsky E, Soni S, Veres G, Negre O, Ross RW, Davidson D, Petrusich A, Sandler L, Asmal M, Hermine O, De Montalembert M, Hacein-Bey-Abina S, Blanche S, Leboulch P, Cavazzana M. Gene Therapy in Patients with Transfusion-Dependent beta-Thalassemia. N Engl J Med. 2018;378(16):1479-1493.
https://doi.org/10.1056/NEJMoa1705342
PMid:29669226

19. Marktel S, Scaramuzza S, Cicalese MP, Giglio F, Galimberti S, Lidonnici MR, Calbi V, Assanelli A, Bernardo ME, Rossi C, Calabria A, Milani R, Gattillo S, Benedicenti F, Spinozzi G, Aprile A, Bergami A, Casiraghi M, Consiglieri G, Masera N, D'Angelo E, Mirra N, Origa R, Tartaglione I, Perrotta S, Winter R, Coppola M, Viarengo G, Santoleri L, Graziadei G, Gabaldo M, Valsecchi MG, Montini E, Naldini L, Cappellini MD, Ciceri F, Aiuti A, Ferrari G. Intrabone hematopoietic stem cell gene therapy for adult and pediatric patients affected by transfusion-dependent ss-thalassemia. Nat Med. 2019;25(2):234-241.
https://doi.org/10.1038/s41591-018-0301-6
PMid:30664781

20. Bauer DE, Kamran SC, Lessard S, Xu J, Fujiwara Y, Lin C, Shao Z, Canver MC, Smith EC, Pinello L, Sabo PJ, Vierstra J, Voit RA, Yuan GC, Porteus MH, Stamatoyannopoulos JA, Lettre G, Orkin SH. An erythroid enhancer of BCL11A subject to genetic variation determines fetal hemoglobin level. Science. 2013;342(6155):253-257.
https://doi.org/10.1126/science.1242088
PMid:24115442 PMCid:PMC4018826

21. Bernardo ME, Aiuti A. The Role of Conditioning in Hematopoietic Stem-Cell Gene Therapy. Hum Gene Ther. 2016;27(10):741-748.
https://doi.org/10.1089/hum.2016.103
PMid:27530055

22. Bacigalupo A, Ballen K, Rizzo D, Giralt S, Lazarus H, Ho V, Apperley J, Slavin S, Pasquini M, Sandmaier BM, Barrett J, Blaise D, Lowski R, Horowitz M. Defining the intensity of conditioning regimens: working definitions. Biol Blood Marrow Transplant. 2009;15(12):1628-1633.
https://doi.org/10.1016/j.bbmt.2009.07.004
PMid:19896087 PMCid:PMC2861656

23. Lucarelli G, Gaziev J. Advances in the allogeneic transplantation for thalassemia. Blood Rev. 2008;22(2):53-63.
https://doi.org/10.1016/j.blre.2007.10.001
PMid:18039551

24. Jacobsohn DA, Duerst R, Tse W, Kletzel M. Reduced intensity haemopoietic stem-cell transplantation for treatment of non-malignant diseases in children. Lancet. 2004;364(9429):156-162.
https://doi.org/10.1016/S0140-6736(04)16628-2

25. Iannone R, Casella JF, Fuchs EJ, Chen AR, Jones RJ, Woolfrey A, Amylon M, Sullivan KM, Storb RF, Walters MC. Results of minimally toxic nonmyeloablative transplantation in patients with sickle cell anemia and beta-thalassemia. Biol Blood Marrow Transplant. 2003;9(8):519-528.
https://doi.org/10.1016/S1083-8791(03)00192-7

26. Walters MC, Hardy K, Edwards S, Adamkiewicz T, Barkovich J, Bernaudin F, Buchanan GR, Bunin N, Dickerhoff R, Giller R, Haut PR, Horan J, Hsu LL, Kamani N, Levine JE, Margolis D, Ohene-Frempong K, Patience M, Redding-Lallinger R, Roberts IA, Rogers ZR, Sanders JE, Scott JP, Sullivan KM, Multicenter Study of Bone Marrow Transplantation for Sickle Cell D. Pulmonary, gonadal, and central nervous system status after bone marrow transplantation for sickle cell disease. Biol Blood Marrow Transplant. 2010;16(2):263-272.
https://doi.org/10.1016/j.bbmt.2009.10.005
PMid:19822218 PMCid:PMC2919571

27. Sadelain M, Riviere I, Wang X, Boulad F, Prockop S, Giardina P, Maggio A, Galanello R, Locatelli F, Yannaki E. Strategy for a multicenter phase I clinical trial to evaluate globin gene transfer in beta-thalassemia. Ann N Y Acad Sci. 2010;1202:52-58.
https://doi.org/10.1111/j.1749-6632.2010.05597.x
PMid:20712772

28. Mansilla-Soto J, Riviere I, Boulad F, Sadelain M. Cell and Gene Therapy for the Beta-Thalassemias: Advances and Prospects. Hum Gene Ther. 2016;27(4):295-304.
https://doi.org/10.1089/hum.2016.037
PMid:27021486 PMCid:PMC4994056

29. Ribeil JA, Hacein-Bey-Abina S, Payen E, Magnani A, Semeraro M, Magrin E, Caccavelli L, Neven B, Bourget P, El Nemer W, Bartolucci P,

Weber L, Puy H, Meritet JF, Grevent D, Beuzard Y, Chretien S, Lefebvre T, Ross RW, Negre O, Veres G, Sandler L, Soni S, de Montalembert M, Blanche S, Leboulch P, Cavazzana M. Gene Therapy in a Patient with Sickle Cell Disease. N Engl J Med. 2017;376(9):848-855.
https://doi.org/10.1056/NEJMoa1609677
PMid:28249145

30. Malik P, Grimley, M., Quinn, C. T., Shova, A., Little, C., L., Lutzko, C., Kalfa, T. A., Niss, O., Mehta, P. A., Chandra, S., Van der Loo, J. C., Grassman, E., Witting, S., Nordling, D., Shreshta, A., Felker, S., C., Reeves, L., Pillis, D., Loberg, A., Bushman, F. D., Knight-Madden, J., Davies, S. M., & Asnani, M. Gene Therapy for Sickle Cell Disease (SCD) Using RVT-1801 Lentivirus Vector and Arulite Reduced Intensity Conditioning Transplant Shows Promising Correction of the Disease Phenotype. American Society of Gene and Cell Therapy; 201; Washington DC.

31. Palchaudhuri R, Saez B, Hoggatt J, Schajnovitz A, Sykes DB, Tate TA, Czechowicz A, Kfoury Y, Ruchika F, Rossi DJ, Verdine GL, Mansour MK, Scadden DT. Non-genotoxic conditioning for hematopoietic stem cell transplantation using a hematopoietic-cell-specific internalizing immunotoxin. Nat Biotechnol. 2016;34(7):738-745.
https://doi.org/10.1038/nbt.3584
PMid:27272386 PMCid:PMC5179034

32. Aiuti A, Naldini L. Safer conditioning for blood stem cell transplants. Nat Biotechnol. 2016;3(7):721-723.
https://doi.org/10.1038/nbt.3629
PMid:27404882

33. Hartigan AJ, Pearse BR, McDonough SM, Proctor JL, Adams HL, McShea MA, Hoban MD, Panwar R, Hyzy SL, Goncalves KA, Palchaudhuri R, Boitano AE, Cooke MP. A Non-Genotoxic Antibody Drug Conjugate Targeting C-Kit for Hematopoietic Stem Cell Transplant Conditioning. Biology of Blood and Marrow Transplantation. 2018;24(3):S47-S48.
https://doi.org/10.1016/j.bbmt.2017.12.598

34. Radtke S, Adair JE, Giese MA, Chan YY, Norgaard ZK, Enstrom M, Haworth KG, Schefter LE, Kiem HP. A distinct hematopoietic stem cell population for rapid multilineage engraftment in nonhuman primates. Sci Transl Med. 2017;9(414).
https://doi.org/10.1126/scitranslmed.aan1145
PMid:29093179 PMCid:PMC6467214

35. Korbling M, Freireich EJ. Twenty-five years of peripheral blood stem cell transplantation. Blood. 2011;117(24):6411-6416.
https://doi.org/10.1182/blood-2010-12-322214
PMid:21460243

36. Falzetti F, Aversa F, Minelli O, Tabilio A. Spontaneous rupture of spleen during peripheral blood stem-cell mobilisation in a healthy donor. Lancet. 1999;353(9152):555.
https://doi.org/10.1016/S0140-6736(99)00268-8

37. Becker PS, Wagle M, Matous S, Swanson RS, Pihan G, Lowry PA, Stewart FM, Heard SO. Spontaneous splenic rupture following administration of granulocyte colony-stimulating factor (G-CSF): occurrence in an allogeneic donor of peripheral blood stem cells. Biol Blood Marrow Transplant. 1997;3(1):45-49.

38. Balaguer H, Galmes A, Ventayol G, Bargay J, Besalduch J. Splenic rupture after granulocyte-colony-stimulating factor mobilization in a peripheral blood progenitor cell donor. Transfusion. 2004;44(8):1260-1261.
https://doi.org/10.1111/j.1537-2995.2004.00413.x
PMid:15265137

39. Lindemann A, Rumberger B. Vascular complications in patients treated with granulocyte colony-stimulating factor (G-CSF). Eur J Cancer. 1993;29A(16):2338-2339.
https://doi.org/10.1016/0959-8049(93)90236-9

40. Pilo F, Angelucci E. Iron Toxicity and Hemopoietic Cell Transplantation: Time to Change the Paradigm. Mediterr J Hematol Infect Dis. 2019 May 1;11(1):e2019030. doi: 10.4084/MJHID.2019.030. eCollection 2019.
https://doi.org/10.4084/mjhid.2019.030
PMid:31205634 PMCid:PMC6548208

41. Fitzhugh CD, Hsieh MM, Bolan CD, Saenz C, Tisdale JF. Granulocyte colony-stimulating factor (G-CSF) administration in individuals with sickle cell disease: time for a moratorium? Cytotherapy. 2009;11(4):464-471.
https://doi.org/10.1080/14653240902849788
PMid:19513902 PMCid:PMC2747259

42. Adler BK, Salzman DE, Carabasi MH, Vaughan WP, Reddy VV, Prchal JT. Fatal sickle cell crisis after granulocyte colony-stimulating factor administration. Blood. 2001;97(10):3313-3314.

https://doi.org/10.1182/blood.V97.10.3313
PMid:11368061

43. De Clercq E. The bicyclam AMD3100 story. Nat Rev Drug Discov. 2003;2(7):581-587.
https://doi.org/10.1038/nrd1134
PMid:12815382

44. Yannaki E, Karponi G, Zervou F, Constantinou V, Bouinta A, Tachynopoulou V, Kotta K, Jonlin E, Papayannopoulou T, Anagnostopoulos A, Stamatoyannopoulos G. Hematopoietic stem cell mobilization for gene therapy: superior mobilization by the combination of granulocyte-colony stimulating factor plus plerixafor in patients with beta-thalassemia major. Hum Gene Ther. 2013;24(10):852-860.
https://doi.org/10.1089/hum.2013.163
PMid:24001178 PMCid:PMC3787462

45. Lidonnici MR, Aprile A, Frittoli MC, Mandelli G, Paleari Y, Spinelli A, Gentner B, Zambelli M, Parisi C, Bellio L, Cassinerio E, Zanaboni L, Cappellini MD, Ciceri F, Marktel S, Ferrari G. Plerixafor and G-CSF combination mobilizes hematopoietic stem and progenitors cells with a distinct transcriptional profile and a reduced in vivo homing capacity compared to plerixafor alone. Haematologica. 2017;102(4):e120-e124.
https://doi.org/10.3324/haematol.2016.154740
PMid:28034992 PMCid:PMC5395121

46. Lagresle-Peyrou C, Lefrere F, Magrin E, Ribeil JA, Romano O, Weber L, Magnani A, Sadek H, Plantier C, Gabrion A, Ternaux B, Felix T, Couzin C, Stanislas A, Treluyer JM, Lamhaut L, Joseph L, Delville M, Miccio A, Andre-Schmutz I, Cavazzana M. Plerixafor enables safe, rapid, efficient mobilization of hematopoietic stem cells in sickle cell disease patients after exchange transfusion. Haematologica. 2018;103(5):778-786.
https://doi.org/10.3324/haematol.2017.184788
PMid:29472357 PMCid:PMC5927997

47. Boulad F, Shore T, van Besien K, Minniti C, Barbu-Stevanovic M, Fedus SW, Perna F, Greenberg J, Guarneri D, Nandi V, Mauguen A, Yazdanbakhsh K, Sadelain M, Shi PA. Safety and efficacy of plerixafor dose escalation for the mobilization of CD34(+) hematopoietic progenitor cells in patients with sickle cell disease: interim results. Haematologica. 2018;103(5):770-777.
https://doi.org/10.3324/haematol.2017.187047
PMid:29419425 PMCid:PMC5927989

48. Tisdale JF, Kanter, J., 2, Mapara, M.Y., Kwiatkowski, J.L., Krishnamurti, L., Schmidt, M., Miller, A.L., Pierciey, F.J., Shi, W.,7, Ribeil, J., Asmal, M., Thompson, Walter, M.C. LentiGlobin Gene Therapy in Patients with Sickle Cell Disease: Updated Interim Results from HGB-206. American Society of Gene and Cell Therapy; 2019; Washington D.C.

49. Richard RE, Siritanaratkul N, Jonlin E, Skarpidi E, Heimfeld S, Blau CA. Collection of blood stem cells from patients with sickle cell anemia. Blood Cells Mol Dis. 2005;35(3):384-388.
https://doi.org/10.1016/j.bcmd.2005.06.014
PMid:16125985

50. Uchida N, Fujita A, Hsieh MM, Bonifacino AC, Krouse AE, Metzger ME, Donahue RE, Tisdale JF. Bone Marrow as a Hematopoietic Stem Cell Source for Gene Therapy in Sickle Cell Disease: Evidence from Rhesus and SCD Patients. Hum Gene Ther Clin Dev. 2017;28(3):136-144.
https://doi.org/10.1089/humc.2017.029
PMid:28447889 PMCid:PMC5695729

51. Pantin J, Purev E, Tian X, Cook L, Donohue-Jerussi T, Cho E, Reger R, Hsieh M, Khuu H, Calandra G, Geller NL, Childs RW. Effect of high-dose plerixafor on CD34(+) cell mobilization in healthy stem cell donors: results of a randomized crossover trial. Haematologica. 2017;102(3):600-609.
https://doi.org/10.3324/haematol.2016.147132
PMid:27846612 PMCid:PMC5394957

52. Ghiaccio V, Chappell M, Rivella S, Breda L. Gene Therapy for Beta-Hemoglobinopathies: Milestones, New Therapies and Challenges. Mol Diagn Ther. 2019;23(2):173-186.
https://doi.org/10.1007/s40291-019-00383-4
PMid:30701409

53. Hanna E, Remuzat C, Auquier P, Toumi M. Gene therapies development: slow progress and promising prospect. J Mark Access Health Policy. 2017;5(1):1265293.
https://doi.org/10.1080/20016689.2017.1265293
PMid:28265348 PMCid:PMC5328344

54. Barde I, Salmon P, Trono D. Production and titration of lentiviral vectors. Curr Protoc Neurosci. 2010;Chapter 4:Unit 4 21.
https://doi.org/10.1002/0471142301.ns0421s53

55. Segura MM, Garnier A, Durocher Y, Ansorge S, Kamen A. New protocol for lentiviral vector mass production. Methods Mol Biol. 2010;614:39-52.
https://doi.org/10.1007/978-1-60761-533-0_2
PMid:20225034

56. Valkama AJ, Leinonen HM, Lipponen EM, Turkki V, Malinen J, Heikura T, Yla-Herttuala S, Lesch HP. Optimization of lentiviral vector production for scale-up in fixed-bed bioreactor. Gene Ther. 2018;25(1):39-46.
https://doi.org/10.1038/gt.2017.91
PMid:29345252 PMCid:PMC5817386

57. Holic N, Seye AK, Majdoul S, Martin S, Merten OW, Galy A, Fenard D. Influence of mildly acidic pH conditions on the production of lentiviral and retroviral vectors. Hum Gene Ther Clin Dev. 2014;25(3):178-185.
https://doi.org/10.1089/humc.2014.027
PMid:25073060

58. Merten OW, Hebben M, Bovolenta C. Production of lentiviral vectors. Mol Ther Methods Clin Dev. 2016;3:16017.
https://doi.org/10.1038/mtm.2016.17
PMid:27110581 PMCid:PMC4830361

59. Ansorge S, Lanthier S, Transfiguracion J, Durocher Y, Henry O, Kamen A. Development of a scalable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures. J Gene Med. 2009;11(10):868-876.
https://doi.org/10.1002/jgm.1370
PMid:19618482

60. Durocher Y, Perret S, Kamen A. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. Nucleic Acids Res. 2002;30(2):E9.
https://doi.org/10.1093/nar/30.2.e9
PMid:11788735 PMCid:PMC99848

61. McCarron A, Donnelley M, McIntyre C, Parsons D. Transient Lentiviral Vector Production Using a Packed-Bed Bioreactor System. Hum Gene Ther Methods. 2019;30(3):93-101.
https://doi.org/10.1089/hgtb.2019.038
PMid:31084376

62. Tinch S, Szczur K, Swaney W, Reeves L, Witting SR. A Scalable Lentiviral Vector Production and Purification Method Using Mustang Q Chromatography and Tangential Flow Filtration. Methods Mol Biol. 2019;1937:135-153.
https://doi.org/10.1007/978-1-4939-9065-8_8
PMid:30706394

63. Manceur AP, Kim H, Misic V, Andreev N, Dorion-Thibaudeau J, Lanthier S, Bernier A, Tremblay S, Gelinas AM, Broussau S, Gilbert R, Ansorge S. Scalable Lentiviral Vector Production Using Stable HEK293SF Producer Cell Lines. Hum Gene Ther Methods. 2017;28(6):330-339.
https://doi.org/10.1089/hgtb.2017.086
PMid:28826344 PMCid:PMC5734158

64. Liu Q. Generation of cGMP-Compliant Stable Packaging and Producer Cell Lines for Inducible Lentiviral Vector Production. American Society of Gene and Cell Therapy; 2019; Washington D.C.

65. Margherita Neri FB, Francesca Rossetti, Manuela Cota, Luca Crippa, Silvia Ungari, Emanuele Simonetti, Luca Allievi, Samuele Corbetta, Federico Lorenzetti, Francesca Bonfanti, Giuliana Vallanti. Lentiviral/Retroviral Vector Large Scale Manufacturing. American Society of Gene and Cell Therapy; 2019; Washington D.C.

66. Sutton RE, Reitsma MJ, Uchida N, Brown PO. Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. J Virol. 1999;73(5):3649-3660.

67. Davis HE, Morgan JR, Yarmush ML. Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes. Biophys Chem. 2002;97(2-3):159-172.
https://doi.org/10.1016/S0301-4622(02)00057-1

68. Davis HE, Rosinski M, Morgan JR, Yarmush ML. Charged polymers modulate retrovirus transduction via membrane charge neutralization and virus aggregation. Biophys J. 2004;86(2):1234-1242.
https://doi.org/10.1016/S0006-3495(04)74197-1

69. Fenard D, Ingrao D, Seye A, Buisset J, Genries S, Martin S, Kichler A, Galy A. Vectofusin-1, a new viral entry enhancer, strongly promotes lentiviral transduction of human hematopoietic stem cells. Mol Ther Nucleic Acids. 2013;2:e90.
https://doi.org/10.1038/mtna.2013.17
PMid:23653154 PMCid:PMC4817938

70. Lanuti M, Kouri CE, Force S, Chang M, Amin K, Xu K, Blair I, Kaiser L, Albelda S. Use of protamine to augment adenovirus-mediated cancer gene therapy. Gene Ther. 1999;6(9):1600-1610.
https://doi.org/10.1038/sj.gt.3300987
PMid:10490770

71. Lee HJ, Lee YS, Kim HS, Kim YK, Kim JH, Jeon SH, Lee HW, Kim S, Miyoshi H, Chung HM, Kim DK. Retronectin enhances lentivirus-mediated gene delivery into hematopoietic progenitor cells. Biologicals. 2009;37(4):203-209.
https://doi.org/10.1016/j.biologicals.2009.01.008
PMid:19264508

72. Delville M, Soheili T, Bellier F, Durand A, Denis A, Lagresle-Peyrou C, Cavazzana M, Andre-Schmutz I, Six E. A Nontoxic Transduction Enhancer Enables Highly Efficient Lentiviral Transduction of Primary Murine T Cells and Hematopoietic Stem Cells. Mol Ther Methods Clin Dev. 2018;10:341-347.
https://doi.org/10.1016/j.omtm.2018.08.002
PMid:30191160 PMCid:PMC6125771

73. Hauber I, Beschorner N, Schrodel S, Chemnitz J, Kroger N, Hauber J, Thirion C. Improving Lentiviral Transduction of CD34(+) Hematopoietic Stem and Progenitor Cells. Hum Gene Ther Methods. 2018;29(2):104-113.
https://doi.org/10.1089/hgtb.2017.085
PMid:29631437

74. Schott JW, Leon-Rico D, Ferreira CB, Buckland KF, Santilli G, Armant MA, Schambach A, Cavazza A, Thrasher AJ. Enhancing Lentiviral and Alpharetroviral Transduction of Human Hematopoietic Stem Cells for Clinical Application. Mol Ther Methods Clin Dev. 2019;14:134-147.
https://doi.org/10.1016/j.omtm.2019.05.015
PMid:31338385 PMCid:PMC6629974

75. Heffner GC, Bonner M, Christiansen L, Pierciey FJ, Campbell D, Smurnyy Y, Zhang W, Hamel A, Shaw S, Lewis G, Goss KA, Garijo O, Torbett BE, Horton H, Finer MH, Gregory PD, Veres G. Prostaglandin E2 Increases Lentiviral Vector Transduction Efficiency of Adult Human Hematopoietic Stem and Progenitor Cells. Mol Ther. 2018;26(1):320-328.
https://doi.org/10.1016/j.ymthe.2017.09.025
PMid:29102562 PMCid:PMC5763075

76. Zonari E, Desantis G, Petrillo C, Boccalatte FE, Lidonnici MR, Kajaste-Rudnitski A, Aiuti A, Ferrari G, Naldini L, Gentner B. Efficient Ex Vivo Engineering and Expansion of Highly Purified Human Hematopoietic Stem and Progenitor Cell Populations for Gene Therapy. Stem Cell Reports. 2017;8(4):977-990.
https://doi.org/10.1016/j.stemcr.2017.02.010
PMid:28330619 PMCid:PMC5390102

77. Petrillo C, Cesana D, Piras F, Bartolaccini S, Naldini L, Montini E, Kajaste-Rudnitski A. Cyclosporin a and rapamycin relieve distinct lentiviral restriction blocks in hematopoietic stem and progenitor cells. Mol Ther. 2015;23(2):352-362.
https://doi.org/10.1038/mt.2014.193
PMid:25270076 PMCid:PMC4312494

78. Wang CX, Sather BD, Wang X, Adair J, Khan I, Singh S, Lang S, Adams A, Curinga G, Kiem HP, Miao CH, Rawlings DJ, Torbett BE. Rapamycin relieves lentiviral vector transduction resistance in human and mouse hematopoietic stem cells. Blood. 2014;124(6):913-923.
https://doi.org/10.1182/blood-2013-12-546218
PMid:24914132 PMCid:PMC4126331

79. Petrillo C, Thorne LG, Unali G, Schiroli G, Giordano AMS, Piras F, Cuccovillo I, Petit SJ, Ahsan F, Noursadeghi M, Clare S, Genovese P, Gentner B, Naldini L, Towers GJ, Kajaste-Rudnitski A. Cyclosporine H Overcomes Innate Immune Restrictions to Improve Lentiviral Transduction and Gene Editing In Human Hematopoietic Stem Cells. Cell Stem Cell. 2018;23(6):820-832 e829.
https://doi.org/10.1016/j.stem.2018.10.008
PMid:30416070 PMCid:PMC6292841

80. Uchida N, Nassehi T, Drysdale CM, Gamer J, Yapundich M, Demirci S, Haro-Mora JJ, Leonard A, Hsieh MM, Tisdale JF. High-Efficiency Lentiviral Transduction of Human CD34(+) Cells in High-Density Culture with Poloxamer and Prostaglandin E2. Mol Ther Methods Clin Dev. 2019;13:187-196.
https://doi.org/10.1016/j.omtm.2019.01.005
PMid:30788387 PMCid:PMC6370599

81. Keever-Taylor CA, Devine SM, Soiffer RJ, Mendizabal A, Carter S, Pasquini MC, Hari PN, Stein A, Lazarus HM, Linker C, Goldstein SC, Stadtmauer EA, O'Reilly RJ. Characteristics of CliniMACS(R) System CD34-enriched T cell-depleted grafts in a multicenter trial for acute myeloid leukemia-Blood and Marrow Transplant Clinical Trials Network (BMT CTN) protocol 0303. Biol Blood Marrow Transplant. 2012;18(5):690-697.
https://doi.org/10.1016/j.bbmt.2011.08.017
PMid:21875505 PMCid:PMC3762249

82. Spohn G, Wiercinska E, Karpova D, Bunos M, Hummer C, Wingenfeld E, Sorg N, Poppe C, Huppert V, Stuth J, Reck K, Essl M, Seifried E, Bonig H. Automated CD34+ cell isolation of peripheral blood stem cell apheresis product. Cytotherapy. 2015;17(10):1465-1471.
https://doi.org/10.1016/j.jcyt.2015.04.005
PMid:25981397

83. Aleksandrova K, Leise J, Priesner C, Melk A, Kubaink F, Abken H, Hombach A, Aktas M, Essl M, Burger I, Kaiser A, Rauser G, Jurk M, Goudeva L, Glienke W, Arseniev L, Esser R, Kohl U. Functionality and Cell Senescence of CD4/ CD8-Selected CD20 CAR T Cells Manufactured Using the Automated CliniMACS Prodigy(R) Platform. Transfus Med Hemother. 2019;46(1):47-54.
https://doi.org/10.1159/000495772
PMid:31244581 PMCid:PMC65