REVIEW ARTICLE

Gene Therapies for Transfusion-Dependent β-Thalassemia

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β-Thalassemia is one of the most prevalent monogenic diseases usually caused by quantitative defects in the production of β-globin, a component of adult hemoglobin (α2β2), leading to severe anemia. Technological advances in genome sequencing, stem cell selection, viral vector development, transduction and gene-editing strategies now allow for efficient *ex-vivo* genetic manipulation of human hematopoietic stem cells that can lead to a meaningful clinical benefit in thalassemia patients. In this perspective, the status of the gene-therapy approaches available for transfusion-dependent thalassemia and early results of clinical trials are discussed. It is highly anticipated that gene therapies will soon become a treatment option for patients lacking compatible donors for hematopoietic stem cell transplant and will offer a suitable alternative for definitive treatment of β-thalassemia, even in young children.

Keywords: *BCL11A, Gene-editing, Hematopoietic stem cell transplantation, Lentivirus vectors.*

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β-thalassemia is a monogenic disorder characterized by reduced or absent synthesis of the β-globin chain, one of the main components of adult hemoglobin (HbA, α2β2). Several hundred mutations (both point mutations and deletions) are now described in the human β-globin gene (*HBB* gene cluster on chromosome 11, **Fig. 1**) or its regulatory elements, leading to decreased (β^+ genotype) or absent (β^0 genotype) synthesis of the β-globin [1]. This results in a relative increase in the unattached α chains (α/β-chain imbalance) that form insoluble hemi-chromes in the erythrocyte progenitors. The hemi-chromes damage the erythrocyte membrane, leading to severe intramedullary erythrocyte apoptosis (ineffective erythropoiesis) and severely shortened red blood cell (RBC) life span due to extra-medullary hemolysis, leading to severe anemia (low hemoglobin, Hb) [2,3].

The phenotype of β-thalassemia is variable depending upon the reduction (α^+/β^+) or complete absence of β-globin chain synthesis (β^0/β^0) and other genetic variables like co-inheritance of α- and γ-mutations, as well as co-inheritance of other hemoglobinopathies (e.g. HbE, Lepore and sickle hemoglobin) [4,5]. Some mutations also alter the fetal hemoglobin (HbF, α2γ2) to HbA switch and may lead to higher production of HbF into adulthood (hereditary persistence of fetal Hb, HPFH) resulting in less severe anemia [6,7]. Therefore, though the severity of thalassemia can be usually predicted based on the mutation analysis of the *HBB* cluster, other genetic factors may modify the actual phenotype and transfusion requirements.

Although the switch from γ- to β-globin synthesis begins before birth, complete replacement of the HbF by HbA occurs in the postnatal period. Consequently, infants with severe β-globin chain abnormality become transfusion-dependent around 6 months of age, when levels of HbF decrease significantly. Based on their transfusion needs, β-thalassemia patients are classified as transfusion-dependent thalassemia (TDT) or non-transfusion-dependent thalassemia (NTDT), although these definitions are also fluid, as some NTDT patients may need regular transfusions as they become older [8].

β-thalassemia has a high global incidence, especially in Asia (northern and eastern India) and Eastern Mediterranean regions. The conventional management of patients affected by the severe form of the disease relies on chronic and regular blood transfusions (every 3-4 weeks) to maintain nadir hemoglobin at or above 9 g/dL

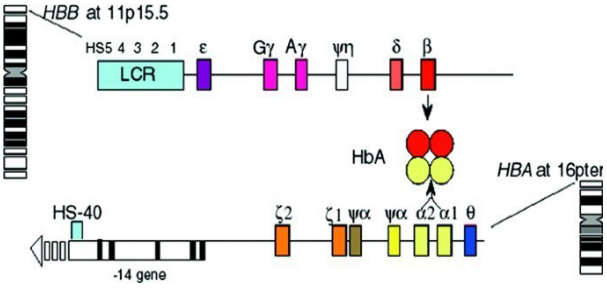

Fig. 1 *HBB* gene cluster (β-globin gene) and *HBA* gene cluster (α-globin gene); transcribed globin proteins combine to make adult hemoglobin (HbA, ∝2β2).

along with iron chelation therapy, to prevent the toxicities of iron overload [3,9].

Currently, the only curative therapy is allogeneic hematopoietic stem cell transplant (HSCT) from an HLA-matched sibling or unrelated donor or cord blood unit (located through national bone marrow donor registries), with good outcomes [10]. HSCT is recommended in relatively younger patients, prior to development of increased iron overload in organs (especially, liver and myocardium) and when suitable HLA-matched donors are available to decrease the risks of toxicity and graft-versus host disease (GVHD). Disease-free survival exceeds 85%, depending on patients' age, HLA-matching and clinical factors like iron overload, liver fibrosis and hepatomegaly [11,12]. Full matched sibling donor HSCT in younger children (<16 years of age) is considered standard of care, while alternative donor HSCT (from mis-matched unrelated or haploidentical donors) are still experimental, and are not devoid of complications like rejection, viral reactivations, and graft versus host disease (GVHD) [13-15].

GENE THERAPY FOR THALASSEMIA

The era of genome sequencing, understanding of the *HBB* gene cluster and its strict regulation and control, along with advancements in vector development and gene-editing platforms, has provided new options for the treatment for thalassemia patients. The expression of β-like genes is regulated by a locus control region (LCR) via looping-mediated interactions with the globin promoters, therefore these LCR and promoter regions are essential for the globin gene expression [16]. The complete understanding of the switch from γ-globin to β-globin production during infancy, and the control of this switch by various transcription factors (TF) has provided new targets for gene-modifications. Speckle-type POZ protein (SPOP), globin transcription factor 1 (GATA-1) and B-cell Leukemia/Lymphoma 11A (BCL11A) are now recognized as important TFs, that bind to specific sites in the *HBB* gene and control the switch from production of HbF to HbA [17-19].

Advances in vector development, transduction of human stem and progenitor cells (HSPCs) and various gene-editing tools, provide a new hope for availability of curative options soon, making gene-therapy one of the most promising treatment options.

The goal of current gene therapy strategies is to induce production of β- or γ-globin, thereby decreasing the levels of unattached α-globin chains, to restore the alpha/non-alpha globin ratio in RBCs. This should lead to correction of ineffective erythropoiesis and improved RBC lifespan (decreased hemolysis), with larger number of erythrocytes with higher hemoglobin surviving longer

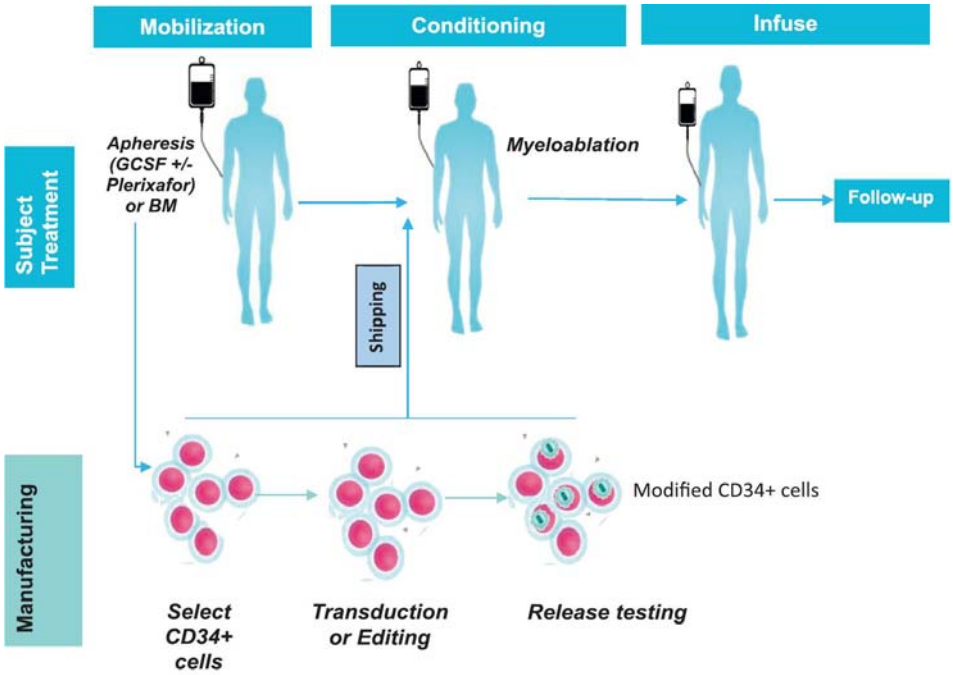

Fig. 2 Overview of treatment plan for gene-modified human stem and progenitor cells.

in the peripheral blood, leading to the correction of anemia and reduction in transfusion needs [20-23].

The common treatment schema of patients undergoing autologous gene modification and infusion is shown in **Fig. 2**. Major steps in the treatment are explained below.

Stem cell mobilization and collection: G-CSF and plerixafor mobilized HSPCs (HPC-Apheresis) are obtained in TDT patients by apheresis procedure as the starting material for gene-modification. Plerixafor is added upfront in the collection protocols, as it leads to efficient mobilization of large number of stem cells in the periphery and decreases the number of collection days and procedures needed for adequate number of stem cells to be collected for gene modifications [24,25]. Adequate HSPCs can be collected from the TDT patients using this combination, despite G-CSF dose reductions recommended in post-splenectomy patients to avoid hyperleukocytosis. HSPCs are collected via the leukapheresis procedure and large volumes of blood (~15-20 L of recirculated blood volume for adults or approximately four total blood volumes in younger patients) should be cycled per day, based on patient tolerability. Average HPC-A collections over 2-3 days in thalassemia patients can yield approximately 15-50×10^6 CD34+/kg (based on experience from early clinical trials) which are adequate for manufacturing and for storing a small fraction (≥2 ×10^6 CD34+/kg cells) as an unmanipulated 'back-up', as a safety precaution in case of non-engraftment with modified HSPCs. The collected HSPCs undergo CD34+ enrichment process, prior to undergoing gene-modification.

Myeloablation: Efficient myeloablation of the expanded erythroid pool in the bone marrow of the TDT patients is essential to create adequate space in the bone marrow niches for adequate engraftment of gene-modified HSPCs, as the gene-modified HSPC do not have a selective survival advantage in thalassemia over the non-gene-modified HSPC. Busulfan is currently the best agent to achieve myeloablation, as the dose can be tailored for each patient based on first dose pharmacokinetics to achieve a standardized target dose range required for myeloablation and to avoid excessive extra-medullary toxicity and lymphodepletion.

Infusion of gene-modified stem cells: The gene-modified HSPCs are usually cryopreserved in 5% dimethyl sulfoxide (DMSO) solution. Once the final product meets all the release criteria (sterility, viability, purity, and % gene-editing frequency or vector copy number [VCN] for gene-insertions), and minimum cell dose criteria (> 2-3× 10^6 CD34+/kg) needed for hematopoietic engraftment, the cryopreserved cells are transported to the treatment site. The cells are thawed and infused intravenously as per standard infusion procedure for autologous stem cell transplants.

Post-transplant care: Care in a specialized BMT unit is recommended, as these patients do become neutropenic and need transfusion support (packed red blood cells and platelet transfusions). Close monitoring and supportive care for busulfan related side-effects, especially mucositis, nausea, infections and veno-occlussive disease (VOD) of liver is recommended. Patients are discharged once they achieve neutrophil engraftment, can eat, drink, and retain their prophylactic medications. Since busulfan is myeloablative but does not cause severe lymphopenia, infection prophylaxis is only recommended for a short period post-transplant. Currently, a 15 years follow up is required for all gene-therapy trials as dictated by regulatory agencies in US and EU. This long follow up is required to ascertain the durability and safety of these experimental approaches.

Currently, the gene-therapy approaches can be divided into two broad groups viz., gene-insertion, and gene-editing approaches.

Gene-Insertion

This involves insertion of a lentiviral or retroviral vector, that contains the whole regulatory machinery and the β-or γ-globin producing genes, into autologous HSPCs 'ex-vivo', and then infusing these modified HSPCs back to the patient after myeloablation [26-28]. Though conceptually straightforward, the field has techno-logically advanced only recently, where the vectors (packaged with the large *HBB* gene and its regulatory elements- promoter, enhancer and parts of LCR) can now be produced at a large scale, achieve high levels of purification and potency to transfect large number of 'non-proliferating' human stem cells to provide clinical meaningful responses [22,29]. For a long-lasting correction and life-long production of erythrocytes (with the hope of one-time curative treatment), the insertions are done in HSPCs (CD34+ enriched population, Milteyni), which includes the long-term repopulating subsets of stem cells. For gene insertion into stem cells, the globin producing genes are placed under the control of an erythroid-specific promoter, so that the transcription of the inserted genes can only occur in erythroid precursors, and not in white blood cells or platelets, which are also derived from the modified hematopoietic stem cells [30].

There are multiple designed lentiviral vectors in clinical trials now for β-thalassemia (**Web Table I**). Once a significant number of HSPCs have been transduced and infused back to a patient, it is expected that the

erythrocyte progenitors derived from these modified stem cells will produce enough β- (or γ) globin (depending on the insertion) to combine with α-chains and reduce the α/β imbalance.

Risks of Gene-Insertion

Since the vector insertions into the stem cells occur randomly and remains largely an uncontrolled process, there is a small risk that some insertions into human stem cells can occur near proto-oncogenes and can stimulate clonal proliferation leading to leukemia/myelodysplastic syndrome (MDS) [31-33]. With the new optimized and self-inactivating (SIN) lentiviral vectors, the insertions into the human stem cells occur 'semi-randomly' i.e. lentivirus insertions occur at preferential sites in the transcription units of human genome, but still lead to polyclonal reconstitution, compared to retroviral vectors that were associated with high risk of insertional mut-agenesis [34]. All clinical trials currently perform integration site analysis to monitor patients of any emerging clonal population. Currently, regulatory agencies require all patients treated with gene therapies to be followed for a total period of 15 years, to clearly establish the incidence of this risk. Fortunately, till date, none of the patients treated with lentiviral vectors have developed any leukemia or MDS related to lentiviral vector insertions [35].

Results of gene-insertion clinical trials: All patients treated recently have tolerated the conditioning regimen with myeloablative doses of busulfan without any unexpected toxicity. Approximately 10% of patients are reported to have developed mild to moderate veno-occlusive disease (VOD) of the liver related to underlying liver fibrosis but have responded to supportive care or defibrotide treatment. In the early Phase 1/2 trials, all patients had engrafted, though efficacy analysis of the first few patients treated with BB305 lentiviral vector, showed variable responses and total hemoglobin production. This variability is expected, as patients with β-thalassemia have large genetic heterogeneity due to varied mutations in the *HBB* cluster and various genetic modifiers and therefore, the level of hemoglobin required to become transfusion independent is variable. The initial results of two concurrent trials (HGB 204 and 205 using BB305 vector), show an average production of 4-5 g/dL of HbA^{T87Q} from the gene-insertions (HbA^{T87Q} is the gene-insertion derived HbA that can be detected separately from transfusion derived HbA by HPLC due to presence of one amino-acid substitution: Threonine at 87 position instead of Glutamine) [30]. An increase of hemoglobin by ~5 g/dL is enough to lead to transfusion independence in HbE/β-thalassemia and β^0/β^+ patients, but only leads to decrease in transfusion requirements in β^0/β^0 patients, where there is a need for higher levels of hemoglobin production to become transfusion independent [22]. Ninety percent (18/20 with >3 months follow up) of non-β^0/β^0 patients treated show rapid rise in gene-derived hemoglobin (HbA^{T87Q}) production post-treatment, maintaining total hemoglobin levels of >9 g/dL (mean 11.6 g/dL; range 9.3-13.3g/dL), with transfusion independence [36]. Based on early encouraging results and safety profile, the lentiglobin gene therapy (Zynteglo) was conditionally approved in EU in June, 2019 for TDT patients with non-β^0/β^0 genotype who are ≥12 years of age (this is still not approved by FDA in US). The results for the β^0/β^0 patients are still under study (HGB 212 trial, NCT 03207009), but do show variable results with 8/11 patients followed for >3 months maintaining hemoglobin above 9 g/dL, though it is still early to comment on durability of the outcomes at this stage [37].

Gene-Editing

Availability of new tools and techniques in the last few years is leading to a rapid development of gene-editing approaches to ameliorate the anemia in thalassemia patients. Last few years have seen advances in availability of different engineered nucleases – zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALENS), and clustered regularly interspaced short palindromic repeats (Crispr)-associated-nuclease 9 (Crispr-Cas9), which are nucleases that act like mole-cular scissors and cut the human DNA at precise locations [38-40]. These nucleases differ in their precision, specificity, efficiency, and ability to make single versus double stranded edits in the target sequence of DNA. The major differences between gene-insertion and gene- editing platforms are highlighted in **Table I**.

Of these techniques, Crispr-Cas9 is the most appealing, as it leads to precise double stranded breaks in the DNA helix, using a pre-designed 42-nucleotide guide sequence (Crispr guide), which has bases complimentary to the target site of the desired break in the DNA [41-43]. The guide carries the Cas-9 nuclease to the target location in the genome to make small edits. Electroporation of Cas9 nuclease and single guide RNA (sgRNA) as a ribonucleo-protein (RNP) complex leads to efficient delivery of genome editing material into HSPCs [44].

BCL11A (the TF that controls the switch from HbF to HbA and functions as a repressor of HbF) provides an excellent target for gene-editing approaches for hemo-globinopathies [45,46]. By suppressing BCL11A TF, it is postulated that HbF production can be triggered again in thalassemia patients to a sufficient degree to ameliorate anemia and avoid transfusions.

Table I Pros and Cons of Gene-Insertion vs Gene-Editing

Parameter	*Gene insertion*	*Gene- editing*
Insertions/Edits	Semi-random	Precise edits
Efficiency	Moderate	High for NHEJ
Therapeutic efficacy based on	Transduction efficiency (VCN)	In-del and NHEJ efficiency
MOA	Produce HbA or F	Produce HbF or Recreate HPFH
Delivery	Lentivirus vectors transduction	Extrapolation of Crispr- Cas9 nuclease + sgRNA or mRNAs encoding specific ZFN
Regulation of gene expression	Vector to provide promoters and regulatory elements of the *HBB* gene	Uses endogenous regulation
Safety	• Recombinant HIV • Insertional oncogenesis • Safety data can be generalized for all lentiviral vectors	• No risk of HIV • off-target' activity • Safety data is specific for the guide/ mRNA and cannot be generalized
Cost of goods	High (viral vector manufacturing)	Low (for NHEJ)

VCN-vector copy number (average insertion of the gene in HSPCs); In-del-small insertions-deletions at the site of edit that can be analyzed by PCR as a marker of editing efficiency; NHEJ-non-homologous end-joining; MOA-Mechanism of Action; sgRNA-specific guide RNA (Crispr); off-target-an unintended site of gene-edit, especially in a functional gene, ZFN-zinc finger nucleases.

Making specific deletions in the erythroid specific enhancer region of the *BCL11A* gene is a promising approach that is being explored currently [47,48]. Two programs to treat TDT are using either ZFN or Crispr-Cas9 platforms to make small deletions in the erythroid specific enhancer region of the *BCL11A* gene located on Chromosome 2. The major advantage of these platforms is that they do not directly make edits in the *HBB* gene, as they target the *BCL11A* gene, allowing the endogenous regulation and sustained production of the globin proteins to continue. These clinical trials are currently recruiting patients.

Another approach to increase HbF production is to recreate the mutations seen in patients with HPFH by making gene edits in the *HBB* gene. This is achieved by: *i*) creating small deletions e.g. in the γ-δ intergenic region leads to significant enhancement of the γ-gene expression [6]; *ii*) creating small deletions in the area of *HBB* cluster where BCL11A binds (e.g. CCAAT box region), so the effect of TF can be inhibited [49,50]; and *iii*) creating point mutations in the β-globin promoter region that can also lead to over expression of the mutated gene [51].

Pre-clinical studies are currently ongoing using Crispr-Cas12 platform to perform edits in the CCAAT box of the *HBB* gene, which overlaps with the BCL11A TF binding site, to increase the levels of HbF. This approach requires a higher degree of precision ('on-target' activity), so as not to disrupt the endogenous production of globin proteins. Both the gene-insertion and gene-editing methods, now scaled to human applications, are in multiple clinical trials now (**Web Table I**).

Pros and Cons of Gene-Editing Strategy

The main advantage of gene-editing (especially Crispr-Cas9 or Cas12) platform is the high efficiency and precision of the gene-edits made in the defined DNA locus [52].The main drawback of gene-editing nucleases is that they can make unintended edits in other parts of the genome, what is called 'off-target' activity [53,54]. Despite their design for accurate target gene editing, unintended off-target interactions between nucleases and genome sequences can still occur. There are multiple cell based and in-vitro assays and computational strategies designed to assess the off-target activity of the guides and nucleases and to predict their functional importance during pre-clinical assessments [55-58]. The goal of these pre-clinical assessments is to define the efficiency of 'on-target' editing and ascertain risks of 'off-target' activity (if any) of a Crispr guide.

In addition to potential off-target activity, chromosomal rearrangement events can also occur, due to double stranded breaks induced during gene-editing [59]. Therefore, serial karyotype analysis is also important during follow-up to analyze chromosome instability of gene-editing platforms.

The assessment of on-target, off-target and genotoxicity assays done in the gene-editing platforms is specific to the guide and the nuclease used to make the gene edits. Unlike the gene-insertion trials using

lentiviral vectors, the safety profile of the gene-editing techniques cannot be generalized, as it is specific for the guide and nuclease. Therefore, it is essential to keep this caveat in mind when comparing adverse events of one gene-editing clinical trial with another.

Long-term assessments of safety in clinical trials is still the gold standard compared to the computational models for analyzing off-target activity currently available [55-57], as detection of an 'off-target' site activity for a guide does not necessarily mean it will lead to a clinically meaningful adverse event.

Results of gene-editing clinical trials: Gene editing is currently undergoing phase I trials in humans. The results of the first patient (β^0/IVS-1-110 genotype) treated with Crispr-Cas9 gene editing (CTX001 product) at 12-months post-treatment show that the patient is transfusion independent, with total hemoglobin level of 12.7 g/dL (12.4 g/dL of Hb-F), and 99% erythrocytes in peripheral blood expressing high levels of HbF (F cells) [60].

Therefore, it is essential to recognize that long-term safety, durability, with continued transfusion independence and improvement in quality of life with no further requirement for chelation therapy, will decide which of these platforms lead to optimal risk-benefit ratio for acceptability.

PEDIATRIC PERSPECTIVES

Most of the Phase I human clinical trials of gene therapy are initiated first in adult patients (>18 years of age) who can understand the risks and benefits of these approaches clearly and consent to the experimental treatment. Once safety is established in the initial cohort of adult patients, the age can be lowered to include younger patients. Currently, lentiviral gene insertion trials are enrolling patients >12 years of age and the goal is to follow similar regulatory strategy for other gene-editing trials, once the initial safety data is available. The younger age limit needs to be established, as it is not the busulfan toxicity, but the risks of HSPCs collection via apheresis procedures in very young patients (currently safety is established for patients >20 kg without requiring any blood priming or other safety precautions).

Since the 'off-target' effects of many of these gene-editing strategies and the risk of insertional oncogenesis may require a longer duration of follow-up in pediatric patients to establish safety, therefore it is expected that for many of these new experimental trials it may take longer time for safety to be established prior to approval in younger patients.

It is also expected that younger patients may tolerate busulfan myeloablation much better than older patients with organ dysfunctions related to iron overload, although the issue of fertility cryopreservation needs to be discussed with individual families as part of the consent process (as infertility is a common long-term toxicity of busulfan and sperm or egg cryopreservation options may be limited in younger patients compared to adults). It is to be noted that the risks of infertility also exist with allogeneic HSCT where chemotherapy based conditioning regimens are utilized.

It is also important to note that correction of ineffective erythropoiesis is an important treatment goal for young patients, other than transfusion independence, to avoid complications of NTDT later in life.

Hence, it is envisioned that gene therapy may provide an alternative option of treatment for younger patients with TDT, especially in patients who lack well matched (HLA) family donors and in countries where large national bone marrow donor registries or cord blood banks do not exist.

CONCLUSIONS

Recent advances in whole genome sequencing, an understanding of the control and regulation of *HBB* gene along with improvements in vector biology and manufacturing, availability of new gene-editing nucleases that can lead to sufficient degree of gene modifications in HSCs to achieve meaningful clinical benefit, has recently led to multiple active clinical trials in patients. The early data from these experimental trials looks promising with potential to lead to a long-term durable transfusion independence and one therapy has already been approved in EU for TDT patients >12 years of age for non-β^0/β^0 patients. There is a hope that with the continued analysis of safety, durability and with continued refinement of manufacturing with improved efficiencies, gene therapies could potentially address the global health burden of β-thalassemia.

Note: Supplementary material related to this study is available with the online version at *www.indianpediatrics.net*

Competing interests: The author is also employed by Crispr Therapeutics Inc. that sponsors the CTX001 thalassemia trial. Only publicly available information has been provided and the manuscript was not influenced in any way by this relationship. Part of the text in this manuscript was adapted for pediatrics audience from previously submitted reviews to other journals by the author.

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Web Table I Listing of Current Gene Therapy Clinical Trials for TDT in North America and EU

Trial NCT number	*Sponsor*	*Phase*	*Patient population*	*Vector or platform/DP name*	*MOA*	*References*
NCT 01745120 and 02151526	bluebird bio (HGB-204 and 205)	1/2	All TDT	SIN Lentiviral vector, BB305 ($\beta^{A\text{-}T87Q}$)	Increase HbA^{T87Q} levels	Thompson et.al, NEJM [24]
NCT 02906202	bluebird bio (HGB-207)	3	TDT with non-β^0/β^0 genotypes	SIN Lentiviral vector, BB305($\beta^{A\text{-}T87Q}$) with process improvements	Increase HbA^{T87Q} levels	Thompson et.al, 2019 [32]
NCT 03207009	bluebird bio (HGB-212)	3	β^0/β^0 genotypes	SIN Lentiviral vector, BB305($\beta^{A\text{-}T87Q}$) with process improvements	Increase HbA^{T87Q} levels	Lal et.al, 2019 [33]
NCT 02453477	IRCCS, San Rafael and Orchard Therapeutics	1/2	All TDT (6-35 years)	SIN Lentiviral vector (GLOBE)-β^A-globin; OTL-300	Increase HbA levels	Marktel et.al, [51]
NCT 03432364	Bioverative and Sanofi	1/2	All TDT (18-40 years)	Gene-editing of erythroid specific region of BCL11a with ZFN; ST 400	Increase HbF	No publication
NCT 03655678	Crispr Therapeutics and Vertex (CTX001-111)	1/2	All TDT (18-35 years)	Crispr-Cas9 gene-editing of erythroid specific BCL11a region; CTX001	Increase HbF	Corbaciogl u et.al, 2020 [60]
Pre-clinical	Editas Medicine	TBD	TDT	Crispr-Cas12 gene-editing of β-globin locus that binds BCL11a	Recreate HPFH	No publication