Novel therapies in β- thalassemia

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Abstract

Beta-thalassaemia is one of the most significant haemoglobinopathies worldwide resulting in the synthesis of little or no β-globin chains. Without treatment, β-thalassaemia major is lethal within the first decade of life due to the complex pathophysiology which leads to wide clinical manifestations. Current clinical management for these patients solely relies on repeated transfusions followed by iron chelating therapy which can eventually results into multi-organ damage. A number of novel approaches to correct the resulting α/β globin chain imbalance are currently being developed. These include reactivation of foetal haemoglobin by pharmacological compounds, allogenic hematopoietic stem cell transplantation (HSCT) and gene therapy. Up to now, the only curative treatment for beta-thalassemia is HSCT, but this is a risky and costly procedure. Gene therapy either by gene addition or gene editing is emerging as a powerful approach to treat this disease. Gene addition is currently based on transplantation of autologous hematopoietic stem cells genetically modified with an integrating lentiviral vector expression the globin gene while gene editing involves the use of CRISPR/Cas9 to correct the causative mutation. Although the early outcomes of the clinical trials in gene therapy are showing promising results, they have also highlighted a number of limitations. In this review we will discuss about the current management strategies used to treat beta-thalassaemia and also focus on novel therapies.

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Abstract

Beta-thalassaemia is one of the most significant haemoglobinopathies worldwide resulting in the synthesis of little or no β-globin chains. Without treatment, β-thalassaemia major is lethal within the first decade of life due to the complex pathophysiology which leads to wide clinical manifestations. Current clinical management for these patients solely relies on repeated transfusions followed by iron chelating therapy which can eventually results into multi-organ damage. A number of novel approaches to correct the resulting α/β globin chain imbalance are currently being developed. These include reactivation of foetal haemoglobin by pharmacological compounds, allogenic hematopoietic stem cell transplantation (HSCT) and gene therapy. Up to now, the only curative treatment for beta-thalassemia is HSCT, but this is a risky and costly procedure. Gene therapy either by gene addition or gene editing is emerging as a powerful approach to treat this disease. Gene addition is currently based on transplantation of autologous hematopoietic stem cells genetically modified with an integrating lentiviral vector expression the globin gene while gene editing involves the

use of CRISPR/Cas9 to correct the causative mutation. Although the early outcomes of the clinical trials in gene therapy are showing promising results, they have also highlighted a number of limitations. In this review we will discuss about the current management strategies used to treat beta-thalassaemia and also focus on novel therapies.

Introduction

Beta-thalassaemia is one of the most common genetic blood disorders worldwide, caused by a spectrum of mutations that results in a quantitative reduction of structurally normal β-globin chains. It is most prevalent in the Mediterranean region, parts of North and sub-Saharan Africa, the Middle East, Indian subcontinent and Southeast Asia and it is estimated that 68,000 babies are born each year suffering from this disease [1], [2].

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Downregulation of β-globin gene (*HBB*) can be caused by a whole spectrum of mutations. In fact, more than 400 mutations in the β-globin gene have now been described ranging from point mutations to small deletions limited to the *HBB* to extensive deletions of the whole β globin cluster (*https://ithanet.eu/db/ithagenes?action=list&hem=2*). The non-deletional mutations include single base substitutions, small insertions or deletions of one to a few bases located within the gene or its immediate flanking sequences. These mutations can affect every stage of β-globin expression, from transcription to RNA processing to translation. Although β-thalassaemia is rarely caused by deletions, a number of deletions that are restricted to the *HBB* gene itself have been described. These deletions range from 25bp up to 6kb [3]. Subjects with a single β-thalassaemia allele are called β-thalassaemia traits or carriers and they are usually asymptomatic. Such individuals present with a mild hypochromic, microcytic anaemia and elevated HbA_2. When both β globin gene alleles are affected, no β globin is produced and these patients are referred to as β-thalassaemia homozygotes or major. β-thalassaemia major presents itself within the first 2 years of age with severe anaemia, poor growth and skeletal abnormalities if left untreated [4]

Due to the imbalance in globin chain synthesis, an excess of freed α-globin chains accumulates within erythroid cells. Aggregation, denaturation and degradation of these excess α-chains leads to the formation of methaemoglobin and insoluble hemichromes resulting in free iron which catalyses the formation of reactive oxygen species. The reactive oxygen species damages the cell membrane leading to ineffective erythropoiesis in the bone marrow and haemolysis of red cells within circulation. This triggers loss of red blood cells in the spleen due to the binding of immunoglobulins and complement components to defective red cell membranes. The resulting severe anaemia leads to reduced tissue oxygenation, increased erythropoietin and bone marrow expansion. This leads to skeletal deformities and osteopenia. Substances released from degenerating red cells increase iron absorption, which contributes to iron overload which can cause liver and heart disease [5],[6].

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Without treatment, β-thalassaemia major is lethal within the first decade of life due to the complex pathophysiology which leads to wide clinical manifestations. Current management strategies for these patients comprise blood transfusion, iron chelation therapy and, for a subset of patients; allogenic bone marrow transplantation also known as haemopoietic stem cell transplantation (HSCT). In recent years, a better understanding of the pathogenesis and clinical effects of the disease has enthused research into a number of promising novel therapies such as therapies that modulate foetal haemoglobin (HbF) induction, gene therapy and gene editing [5], [7], [8]. For this review we will talk about the current management strategies and focus on novel therapies.

Blood Transfusions

Patients with β-thalassaemia major require lifelong, regular blood transfusions administered every 2-5 weeks to maintain haemoglobin levels of at least 9 – 10.5 g/dL. Transfusion is usually started before the age of 2 years and it provides normal red blood cells and suppresses ineffective erythropoiesis, also it enables normal growth and physical activities and helps to reduce hepatosplenomegaly and bone deformities [9]. It is estimated that

approximately 100,000 patients currently receive regular transfusions for beta-thalassemia worldwide and in some countries transfusion therapies can be a huge burden. Although processes for screening, preparation and administration of blood have improved, blood transfusions expose patients to a number of risks such as blood-borne infection, alloimmunization and iron overload [7], [9].

Iron Chelation

In β-thalassaemia patients, iron stores are increased far beyond normal physiological levels primarily due to increased iron absorptions and secondary due to transfusions. Unless effective iron chelation therapy is provided, these patients can suffer from iron overload that affects the heart, liver and endocrine tissues. Three iron chelators are currently approved by the regulatory authorities for the treatment of iron overload in beta-thalassemia [7]. Deferoxamine (DFO), the first commercially iron chelator is a hexadentate iron chelator that binds iron in 1:1 complex. It is administered subcutaneously or intravenously at a dose of 20-50 mg/kg/day. It has a short plasma half-life of 20-30 minutes and therefore it should be administered over a span of 8-10 hours a day on 5-7 days a week [10]. Although the benefits of deferoxamine are well documented, the demanding regime leads to poor compliance.

The introduction of two orally active iron chelators; Deferiprone and Deferasirox were of a great advance in the management of patients with beta thalassaemia. Deferiprone (DFP), the first oral iron chelator was approved in 2011, it is a bidentate iron chelator that forms 3:1 complex. It is usually given at a dose of 75-100mg/kg/day three times daily. DFP may cause gastrointestinal disturbances, increased liver-enzymes, agranulocytosis and neutropenia and therefore patients on DFP should be closely monitored Deferasirox (DFX), the other oral iron chelator is a tridentate that forms 2:1complex. It is given at a dose of 20-40 mg/kg once daily and the most common side effects are gastrointestinal disturbances, rash and mild increases in serum creatinine. Sometimes a combination of both iron chelators is used [7],[11]. Although iron-chelation therapy is available in most countries, death due to iron overload remains an issue in beta-thalassaemia patients.

Haemopoietic Stem Cell Transplantation (HSCT)

Until now the only available curative therapeutic approach for patients with beta-thalassaemia is HSCT. The first HSCT in patients with beta thalassaemia major took place in the 1980s and early 1990s at the transplant centre in Pesaro, Italy. Initially, transplant experiences were limited to good risk young thalassaemia patients with limited morbidities and from matched sibling donors (MSD) [12],[13],[14]. With today advances in medicine, HSCT is not only limited to patients with matched sibling donors but also from unrelated donors and cord blood transplantation [15]. Instead of bone marrow, it was proposed to use peripheral blood stem cell transplantation from HLA-matched siblings but studies showed that this increased risk of cGVHD [16], [17]. In 2003, the possibility of using HLA-identical sibling cord blood for HSCT in thalassaemia major was reported that this type of allograft was associated with decreased risk of both acute GvHD and chronic GvHD [18].

The best clinical outcomes of HSCT among patients with beta thalassaemia are reported in those aged under 14 years at transplantation, in fact in the last decade, almost all transplant centres try to perform HSCT in the first years of life before iron-related complications develop. Experience of HSCT in adult patients is still very limited and only few centres perform HSCT in patients over the age of 18 years. In-fact it is recommended that HSCT in adults is done only in patients who have been well-chelated since infancy. About 25-30% of patients with thalassaemia major have an available MSD but this is not always the case. The experience of HSCT from HLA-disparate relatives is still very limited and the results obtained are inferior than those obtained with HLA-identical siblings as donors. A small study from related donors that were not MSD had a thalassemia major free survival of 94%. This was obtained by using a pre-conditioning phase with hydroxyurea, azathiprone and fludarabine while the conditioning regime included busulfan, thiotepa, cyclophosphamide and rabbit ATG [19], [20]. In case that unrelated donors are used for HSCT, the donor must be selected using high-resolution molecular typing for both HLA class I and II molecules and a stringent criterion of compatibility with the recipient [20].

Although HSCT is the only available curative approach for thalassemia, nevertheless it has been limited by the high cost and significant drawbacks associated with its implementation which include limited availability of MHC-matched donors, the need for long-term immunosuppression and increased risk of immunological complications. Understanding the pathophysiology and clinical effects of beta-thalassaemia has stimulated research into a number of promising therapeutic approaches to tackle this disease.

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Compounds that modulate foetal haemoglobin

After 2 years of age, the main type of haemoglobin present is adult Hb (HbA) together with HbA2 (<3.3%) and small amounts of foetal haemoglobin (HbF, <1). In some individuals, the HbF levels exceed this threshold and genome wide association studies (GWAS) have shown that variant HbF levels are highly inheritable [21],[22]. It is well known that beta-thalassaemia patients with inherited persistent high levels of HbF production have a milder clinical progression than other patients with this disease, and many of these patients do not require transfusions. This is because increased production of γ-globin decreases the α/β-chain imbalance. Reactivation of HbF is one of the novel therapeutic approaches for beta-thalassaemia. Various studies have been carried out to identify quantitative trait loci (QTL) that increases HbF levels to help in the development of clinical HbF inducers.

A number of genomic loci have been identified which include the beta globin (HBB), the HBS1-like translational GTPase-v-myb avain myeloblastosis viral oncogene homolog (*HBS1L-MYB*) on chromosome 6 [23], [24], the B-cell CLL/lymphoma11A (*BCL11A*) locus on chromosome 2 [26] and the Kruppel-like factor (KLF1) on chromosome 19 [27]. The role of *KLF1* in globin gene switching was shown in a large Maltese family with hereditary persistence of foetal haemoglobin (HPFH). Ten out of twenty-seven family members had HbF levels between 3 and 19% which was due to a truncation mutation in the KLF1 gene. Functional work showed that KLF1 have a dual role in the regulation of foetal-to-adult globin gene switching. First, it acts directly on the *HBB* locus as a preferential activator of the*HBB* gene and secondly it acts indirectly by activating the expression of *BCL11A* which, in turns, represses the gamma globin genes [27], [28]. The *MYB* gene is a key regulatory of the balance between proliferation and differentiation during erythropoiesis. MYB inhibits HbF expression through regulating KLF1 [29].

These regulatory transcription factors involved in γ-globin regulation are potential targets for HbF increase. Although a lot of research is focusing in this area, it remains difficult to modulate the function of factors other than enzymes or signal-dependent nuclear factors by disrupting DNA/protein interactions or protein/protein interaction. Furthermore, any interference with erythroid transcription factors may disrupt erythropoiesis, and therefore efforts are being made to design endonucleases capable of precisely disrupt the genomic sequences involved in the expression of gamma globin repressors [30].

One drug that has proved to be clinically effective in some patients with β-thalassaemia is the S-phase cell-cycle inhibitor hydroxyurea (HU). An increase of 2- to 9- fold change in γ-mRNA expression was noted in some thalassaemia patients, but the increases in HbF did not always correlate with an increase in total haemoglobin. [31], [32]. HU treatment of 10-20mg/kg per day has been reported to lead to 40-70% decrease in transfusion needs in thalassaemia major patients. This was mostly noted in individuals with the HbE/β-thalassaemia genotypes [33]. A number of side effects have been reported which include cytopenia, hyperpigmentation, opportunistic infections, marked hypomagnesemia and in about 80% of men azoospermia. Although it is believed that HU is teratogenic, there is little or no risk of leukaemia [34]. Given that reported efficacy was seen in only a subset of patients, and its potential side effects, it is important to identify likely responders and non-responders before initiating treatment. Some studies noted that the increase in HbF levels following HU treatment in β-thalassaemia major patients was correlated with the XmnI polymorphism while in a study of beta-thalassaemia patients of Iran origin, HU response was correlated with *BCL11A* SNPs [35], [36], [37]. Since only a few studies have investigated the correlation between polymorphism and response to HU treatment and conflicting results have been obtained, the criteria for treatment with HU should not be based on these genetic characteristics [38].

Thalidomide, a synthetic glutamic acid derivative, was introduced in the late fifties and was later abandoned because of its teratogenic effect. The first report on its use in thalassaemia patients appeared in 2008 where a 21-year old transfusion dependent woman with beta-thalassaemia major was treated with 100mg/day thalidomide. After 3 months her haemoglobin levels went up from 2.9g/dL to 7g/dL and her HbF went up from 62.3% to almost 100%. In the paper they reported that she was given thalidomide uninterrupted and was never transfused again [39]. In 2017, a centre in India did a retrospective study on 104 thalassaemic patients who received thalidomide between January 2006 and April 2016 to see the effect of thalidomide on ferritin levels. It was found that the ferritin levels reduced to 51% in all patients [40]. In another prospective study between October 2017 and April 2018, 70 known cases of transfusion dependent β-thalassaemia major were given thalidomide at a dose of 2mg/kg to 10mg/kg for 6 months. It was found that thalidomide increased amelotin levels while it reduced ferritin levels in these patients [41]. In another study, a cohort of 37 patients with symptomatic β-thalassaemia were put on low dose Thalidomide (2-10mg/kg) and followed for a minimum of 8 months. In non-transfusion dependent patients, a significant increase in haemoglobin was noted while in transfusion dependent patients, there was a significant drop in yearly transfusions [42]. The risk-benefit of thalidomide still needs to be established and therefore this drug should be administered in the context of clinical studies [43].

Sirolimus is another potential drug for inducing HbF production in thalassaemia patients. Sirolimus is a macrolide compound that acts by selectively blocking the transcriptional activation of cytokines thus inhibiting cytokine production. It is only bioactive when bound to immunophilins and they are potent immunosuppressants and possess both antifungal and antineoplastic properties. Right now, there are two ongoing clinical trials to test Sirolimus in beta-thalassaemia patients. The first trial, NCT03877809 started in June 2019 were 20 beta thalassaemia major patients between the age of 18 and 65 who are on regular transfusion since at least 6 years were recruited. They were administered 0.5mg of Sirolimus daily. After 360 days the HbF will be measured and compared to the HbF before treatment. The second trial, NCT04247750 started in January 2020 and will recruit 15 beta thalassaemia major patients between the age of 18 and 65 and these patients will be administered also 0.5mg of Sirolimus daily.

A number of other non-selective compounds such as cytotoxic compounds, short-fatty acid derivatives and hypomethylating agents are being investigated in-vitro to see their effect on foetal haemoglobin.

Gene therapy

Gene therapy for β-thalassaemia is currently based on transplantation of autologous haemopoietic stem cells (HSCs) genetically modified with integrating viral vectors expressing the transgene of interest [44].The concept of gene therapy for beta-thalassemia emerged a long time ago, in 1978 at the University of California at Los Angeles was included in a plan for the viable treatment for haemoglobinopathies [45]. A number of major technical issues were encountered and early attempts in 1980 to treat beta-thalassaemia patients by inserting the β-globin gene into the bone marrow cells were completely unsuccessful and received a lot of criticism [46]. At that time, the regulatory globin sequences required for high levels of production and efficient methods for gene introduction were not available. After 25 years, these goals were achieved and made possible by the characterization, size reduction, isolation and amalgamation of the β-globin locus regulatory elements and the advent of lentiviral vectors which can transfer complex sequences into hematopoietic stem cells [38]. Currently, the gene-therapy approaches can be divided into two broad groups – (i) gene addition and (b) gene-editing approaches (figure 1)

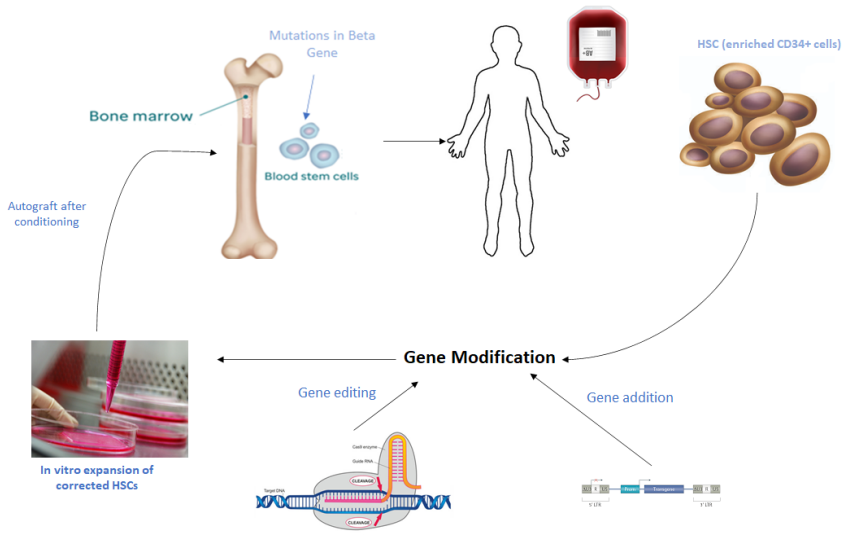

Figure 1: Schematic representation of gene therapy in beta-thalassemia. Mutations in the beta globin gene give rise to beta-thalassaemia. Conventional therapies include transfusion followed by iron chelation therapy. Autologous transplantation of HSCs genetically modified by gene addition or gene editing is another therapy for patients affected by beta-thalassaemia.

Gene-addition

Gene addition involves the insertion of a lenti-viral (LV)/retroviral vector that contains the whole regulatory and the β- or γ-producing genets into autologous human stem cells in-vitro and then infusing these modified stem cells back into the patient. Early gene therapy studies used recombinant γ-retroviral vectors derived from Moloney Leukemia Viruses to introduce the functional copy of the β-globin gene.*In-vitro* , these vectors provided reasonable levels of β-globin transgene expression but *in vivo* , it resulted in a limited variable expression [47], [48], [49]. These types of vectors have a number of defects: (i) instability, (ii) limited cargo capacity and (iii) an inability to transduce non-dividing cells [38]. The use of γ-retroviral vectors was abandoned and in the mid-1990s, lentiviral vectors were derived from human immunodeficiency virus type 1 (HIV1). These types of lentiviral vectors were able to transfer much longer sequences and also were able to transduce cells arrested at the G1-S boundary of the cell cycle [50], [51].

Several studies have shown that self-inactivating lentiviral carrying the human β or γ-globin gene and its fundamental regulatory elements are able to correct the disease in mice with either β-thalassaemia intermedia or major [52], [53], [54]. Several investigators continued to work hard to improve LV in terms of higher transgene expression, acting mostly on regulatory transcriptional components and the addition of insulators to prevent negative chromatin position effects. These included larger HS2 and HS4 elements of the Locus Control Region (LCR) and incorporation of insulator elements and inclusion of HS1 or GATA-1 elements. In most cases, although increased transgenic expression was achieved this happened at the cost of a reduced transduction efficiency and vector copy number/cell [55]. The final approved LVs for clinical trials were the result of an acceptable compromise between good manufacturing yield, title and β-globin expression level [44].

The first clinical trial for β-thalassaemia took place in June 2007 in France, where three patients were treated by gene therapy with the HPV569 LV expressing the β^{T87TQ} globin variant [38]. In this vector, the β-globin gene was under the control of the LCR and the β-globin promoter and was surrounded by tandem copies of the cHS4 core element [56]. The first patient to be treated was an 18-year-old β^{E}/β^{0} who had been transfusion dependent and on parenteral iron chelation since early childhood. Bone marrow cells were processed for CD34+ selection and transduced with the HPV569 LC. After busulfan conditioning the patient

received an infusion of 3.9 x 10^6 cells/kg. After one year, the patient became transfusion independent and his haemoglobin was stable at about 8-9 g/dl after 7 years from gene therapy [57], [58], [59]. Integration site analysis revealed expansion of a single clone in which the provirus was inserted at the high mobility group AT-hook 2. This benign clonal dominance persisted for 9 years after which it started to decline. In 2011, another patient underwent transplantation but the vector copy number was lower and this patient is still transfusion dependent [59].

To achieve higher transduction efficacy the original LV was further improved. This was done by the removal of the cHS4 from HPV569 LV and this yielded

The HGB-204 clinical trial started in 2012 and completed in February 2018. Data from up to 5 years of follow-up from the completed Phase 1/2 as of December, 2019 (ASH, 2019) it was shown that 8 out of 10 treated patients who did not have a β0/β0 genotype achieved and continued to maintain transfusion independency for up to 51.3 months with a median Hb of 10.3 g/dL. In the other two patients, who did not achieve transfusion independency, the transfusion volumes were reduced by 79% and 52%. Three out of 8 patients that were β0/β0 achieved and continued to maintain transfusion independence up to 30.4 months with a median Hb of 9.9g/dL [61].

The clinical trial HGB-207 started in July 2016 and it is estimated to be completed in February 2022. Recruitment is completed, and 23 beta-thalassaemia transfusion dependent who are not β0/β0 genotype between the age of 4 and 34 were transfected with BB305 Lenti-globin after myeloablative conditioning with busulfan. As of June 2020 (EHA, 2020) 19 out of the 23 patients were evaluable for transfusion independence while the other 4 do not have sufficient follow up. Seventeen out of the 19 evaluable patients, achieved transfusion independence with median Hb levels of 11.9g/dL. Improved iron levels were also observed while over half of the patients stopped chelating therapy [62].

The other clinical trial using the BB305 Lenti-globin is HGB-212 which started in June 2017 and it is estimated to be completed by June 2022. As of June 2020 (EHA, 2020) 15 patients with different beta-genotypes were treated and had a median follow up of 14.4 months. Nine out of the 15 were β0/β0, 3 were β0/β+IVS1-110 and 3 IVS1-110 homozygous. Six of eight evaluable patients achieved transfusion independence with median Hb of 11.5 g/dL. 11 out of 13 patients with at least 7 months of follow up did not receive a transfusion [62].

In 2012, a trial opened at MSKCC in New York (NCT01639690) where four beta-thalassaemia transfusion dependent patients were treated with gene therapy using the TNS9.3.55 LV. This protocol differs from the other clinical trials with the BB305 LV because in this trial a reduced intensity conditioning was used rather than a fully myeloablative regimen. This led to insufficient gene marking with minimal clinical benefit [63], [64]. In 2015, the clinical trial NCT02453477 (TIGET BTHAL) started in Italy and it was completed in August 2019. Nine patients with different genotypes (β0/β0, β+/β+ and β0/β+) 3 adults followed by 6 minors were treated. This clinical trial was based on the autologous transplantation of G-CSF and plerixafor mobilized HSCs engineered by the GLOBE lentiviral vector. Following myeloablative conditioning by treosulfan and thiotepa the transduced cells were infused by intraosseous injection. As for April 2019, the three adult patients had reduction of transfusion requirement while 4 paediatric patients are transfusion independent and two are still receiving blood transfusions [65].

The main goal of gene therapy in beta-thalassaemia is to achieve stable introduction of functional globin genes in the patient's own HSCs in order to abolish transfusions by correcting ineffective erythropoiesis and haemolytic anaemia. This can be achieved by taking into consideration a number of critical issues such:

Source of haematopoietic stem/progenitor cells

Nowadays, the preferred source for many autologous and allogenic transplantation approaches is peripheral blood mobilized HSCs. When compared to conventional bone marrow harvest, this minimally invasive procedure provides several-fold higher numbers of HSCs [66], [67]. In adult patients, HSC procurement is critical since the minimal target dose of 2-3 x10^6 CD^{34}cells/kg poses a challenge for steady-state bone-

marrow. Also, favourable gene therapy results are correlated with the dose of transfused cells infused and engrafted [44]. The growth factor granulocyte-colony stimulating factor (G-CSF), was the standard agent used to mobilize HSCs for transplantation. This stimulating factor, results in rapid mobilization within hours following administration [68]. Although it is known to be well-tolerated it raised concerns for its safety in thalassaemia due to the rare events of splenic rupture or thrombosis during mobilization in normal donors and in patients with haematological malignancies [69], [70], [71].

Plerixafor (AMD3100, Mozbil^{TM}) a bicyclam molecule that mobilizes HSCs by selectively and reversibly antagonizing the binding of stromal cell derived factor-1 to chemokine CXC receptor-4 (CXCR4) is considered as an alternative mobilization agent [72]. In conditions resulting in poor mobilization, this agent is approved by FDA and European Medical Agency (EMA) to be used together with G-CSF [73]. A number of mobilization trials took place to define the optimal mobilization approach for adult patients with beta-thalassaemia before implementation of gene therapy. From these trials it was established that the optimal mobilization approach for these patients is either Plerixafor alone or in combination with G-CSF [74], [75], [76], [77].

Dose of transduced HSCs and transduction methods

Two important factors that might affect gene therapy outcome are the transduced cell dose as well as the stem cell source. Determining the dose of the optimal genetically modified cells to transduce still remains unclear. Based on the results available until now from clinical trials we still do not have a direct correlation between transduced cell dose and clinical outcome. In clinical trials, beta-thalassaemia patients have been treated with different cell doses and the vector/copy number in drug products is also variable. Other different variables such as severity of genotype/phenotype, comorbidities, secondary modifiers of the pathology, proportion of engrafting genetically modified long-term analysis may also influence the clinical trial. From clinical trials, preliminary indications show that patients that receive the highest dose of transduce with the highest vector/copy number should have the better outcome [44].

A near-complete transduction of the purified HSCs with LV can be achieved by adding Prostaglandin E_2(PGE_2). In less than 38 hours, it was shown that PGE_2 allows near-complete transduction of HSCs with LV [78]. *In vitro* , the addition of PGE2 was shown to increase VCN and/or transduction efficiency of $CD34^+$ cells [79]. When compared to control transduced cells, addition of PGE2 increased vector transduction of $CD34^+$ cells approximately to 2-fold [80]. Addition of rapamycin which is an inhibitor of the mammalian mTOR pathway also resulted in significantly enhanced transduction without alterations in lentiviral integration profile [81],[82].

Bone-marrow conditioning and HSC administration

Before administration of transduced HSCs, conditioning of the bone marrow must take place. Since beta-thalassaemia is a non-malignant disease, it requires complete elimination of endogenous HSCs to confer therapy. Initially, it was discussed whether a partial or full myeloablation is needed. Non-myeloablation treatment is preferred to conventional fully myeloablation due to the high risk of non-haematological toxicity but it can lead to mixed chimerism. Also, despite the encouraging engraftment rates in mouse-models achieved under non-myeloablative settings with various vectors, this preparative regimen could not be used in vivo [83], [84], [85]. Most of the clinical trials for gene-therapy in beta-thalassaemia nowadays, are using reduced intensity conditioning with Busulfan. The MSKCC trial used Busulfan at 8mg/kg while the other clinical trials with the either HPV569 LV or BB305 LV used Busulfan 12.8mg/kg. The clinical trial of TIGET-BTHAL conditioning was done by Treosulfan 42g/$m^2$and Thiotepa 8mg/kg.

Following myeloablative treatment, administration of the HSCs is usually given by intravenous injection. The systematic delivery via peripheral circulation is an easy procedure for the dissemination of transplanted cells that home and engraft in the bone marrow niche. On the other hand, one of the drawbacks of this administration, is the loss of a significant proportion of injected cells. These cells are lost due to the major filter organs trapping such as the lungs and spleen. The TIGET BTAL clinical trial used an alternative route of administration to overcome cell loss. In this clinical trial the transduced cells were delivered by intraosseous injection bilaterally in the iliac crests [44].

Gene editing

An innovative approach for treating β-thalassaemia is gene editing. In the last decades, several nucleases such as zinc-finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs) were developed for genetic engineering with CRISRP/CAS9 system being the most novel approach. The CRISPR/Cas9 system uses a single or multiple short guide RNAs (gRNA) with 20bp complementary to DNA sequence target. The nucleases will than create site specific double strand breaks which can be repaired either by non-homologous end joining (NHEJ) or by homologous directed repair (HDR) [86], [87].

In-vitro , in the context of β-haemoglobinopathies a number of gene editing strategies have been successful. These include correction of the β-globin gene mutation [88], [89], [90] or induction of endogenous foetal haemoglobin [91]. A recent study used the CRISPR/Cas9 system to mutate a 13-nucleotide sequence in the promoters of the gamma genes. This was done via microhomoglogy-mediated end joining leading to HbF increase to potentially therapeutic levels [92]. Although many labs have been successfully performing gene editing of immortalized cells, gene editing of primary cells remains a challenge due to toxicity caused by gene-editing reagents, off-target mutations and low efficiency of transfection [93].

In the past 5 years, gene correction of patient-specific induced pluripotent stem cells (iPSCs) by CRISPR/CAS9 and cell transplantation has appeared as a promising therapeutic solution for many diseases including β-thalassemia [94], [95]. In 2019, the group of Bauer at the Dana-Farber/Boston Children's Cancer and Blood Disorders Centre at the University of Massachuseets Medical School edited $CD34^+$ HSPCs from seven patients with β-thalassaemia of varying genotypes using CRISPR-CAS9 gene editing. After overcoming technical challenges associated with editing of blood stem cells they have demonstrated that CAS9:single guide RNA ribonucleoprotein (RNP)-mediated cleavage within a GATA1 binding site at the +58 BCL11A erythroid enhancer resulted in a highly penetrant disruption of this motif. The reduced BCL11A expression gave rise to an increase in gamma-globin levels between 35.3 and 75.1% and an increase in gamma protein levels between 27.5 and 46.9% [96]. In the context of beta-thalassaemia two clinical trials one utilizing a CRISPR/CAS9 system and one implementing ZFN are currently in the recruitment phase. The clinical trial NCT03655678 is a phase 1/2 study of the safety and efficacy of a single dose of autologous CRISPR-Cas9 Modified CD34+ HPSPc in 45 subjects with Transfusion-Dependent beta thalassaemia. The other clinical trial, NCT03432364, will assess ST-400 in 6 subjects with transfusion-dependent beta-thalassaemia. ST-100 will be composed of the patient's own blood stem cells which are genetically modified in the lab using Sangamo's zinc finger nuclease technology to disrupt a precise and specific sequence of the enhancer of the BCL11A.

As can be seen from this review, β-thalassaemia has offered a very robust model on which novel therapies and treatments can be explored with a great impact that holds promise on a clinical level. In conclusion, whether an approach to re-induce high levels of HbF in vivo, or a gene editing approach that amount to gene replacement or correction it is now envisaged that such activities are all fruitful approaches to augment HbF levels in individuals with β-type haemoglobinopathies.

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