**Gene therapy using haematopoietic stem and progenitor cells**

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**Abstract**

Haematopoietic stem and progenitor cell (HSPC) gene therapy has emerged as an effective treatment modality for monogenic disorders of the blood system such as primary immunodeficiencies and β-thalassemia. Medicinal products based on autologous HSPCs corrected using lentiviral and gammaretroviral vectors have now been approved for clinical use and the site-specific genome modification of HSPCs using gene editing techniques such as CRISPR/Cas9 has shown great clinical promise. Preclinical studies have shown engineered HSPCs could also be used to cross-correct non-hematopoietic cells in neurodegenerative metabolic diseases. Here, we review the most recent advances in HSPC gene therapy and discuss emerging strategies for using HSPC gene therapy for a range of diseases.

**[H1] Introduction**

Haematopoietic stem cell transplantation (HSCT) has been a routine procedure for treating inborn errors of metabolism and the blood system for over 50 years1,2. The first successful transplantations for the treatment of immune disorders were conducted in 1968, using allogeneic **[G]** stem cells to treat X-linked severe combined immunodeficiency (SCID-X1), an inherited disease caused by inactivating mutations in the gene encoding the interleukin 2 receptor subunit gamma (*IL2RG*), and Wiskott–Aldrich syndrome, a rare X-linked recessive immunodeficiency characterized by thrombocytopenia, eczema and recurrent infections3,4. Extraordinary progress has been made in allogeneic HSCT and it is now used for many genetic diseases in an increasing number of patients. Improvements have been made with regards to donor matching, strategies for more effective control of graft-versus-host disease (GVHD), more effective conditioning regimens and better management of toxicity and infections. However, the availability of immunocompatible donors with optimal human leukocyte antigen (HLA) genotype matching can limit its application5 and morbidity owing to GVHD remains as a result of using unmatched donors. As a result, gene therapy techniques based on the genetic modification of autologous HSPCs have been explored.

Autologous HSPC gene therapy has been investigated for the prevention or treatment monogenic disorders associated with altered blood cell maturation and function, such as diseases of the innate and adaptive immune systems, red blood cell disorders, platelet disorders and bone marrow failure syndromes6-8. HSPC gene therapy techniques to date have employed ex vivo gene transfer, through transduction of the patients’ own HSPCs with a vectors carrying one or more copies of a therapeutic gene7. Once reinfused, genetically-modified HSPCs undergo self-renewal and establish a population of modified cells, which pass the transgene to daughter blood cells on differentiation **(Fig. 1).** The first proof-of-concept HSPC gene therapy studies were conducted in the 1990s to address severe combined immunodeficiency caused by adenosine deaminase deficiency (ADA-SCID); although successful, the efficiency of correction in these studies was low9-12. Improvements in ex vivo culture techniques and new engineering approaches using long terminal repeat (LTR)-driven gammaretroviral vectors have since resulted in clinical benefit in many primary immune deficiencies **[G]**(PIDs)13,14; however, the occurrence of T-acute lymphoblastic leukemia following gene therapy in a significant proportion of patients — caused by insertional mutagenesis into the *LMO2* locus — led to a substantial slowdown of all ex vivo gene therapy approaches15,16. The development of self-inactivating lentiviral vectors as a delivery platform7 has since enabled more effective and safe insertion of therapeutic genes into HSPCs.

Over the past two decades, advances in our understanding of disease biology and gene transfer technology have been translated into remarkable clinical successes (**Fig. 2**). As a result, the field has attracted significant commercial interest and two products have secured regulatory approval by the EMA in Europe — namely Strimvelis for use in ADA-SCID and Zynteglo for use in patients over 12 years old with transfusion-dependent β-thalassaemia (TDT) — with several others expected to follow in both Europe and the United States in the next five years. More than 300 patients have now been treated using HSPC gene therapy in clinical trials and robust evidence for the durability of corrected HSPC treatments and their long-term safety and clinical efficacy have been observed for PIDs including SCID-X1, ADA-SCID, Wiskott–Aldrich syndrome, chronic granulomatous disease **[G]** (CGD), β-thalassaemia, metachromatic leukodystrophy (MLD) and X-linked adrenoleukodystrophy (X-ALD) (Table 1).

Here, we review the most recent advances in HSPC gene therapy. We begin with an overview of the ex vivo gene transfer process, from HSPC collection and genetic modification to engraftment and long-term clonal tracking. We discuss recent technological developments in gene editing, which will help move the field forward to the next generation of medicinal products, broadening the field of application to disorders not amenable to current gene addition approaches. Finally, we discuss the application of HSPC gene therapy for different diseases, including the use of HSPCs as delivery vehicles for therapeutic proteins, and conclude by considering remaining challenges in the field and future perspectives.

**[H1] Ex vivo gene transfer**

***[H2] HSPC collection***

Autologous HSPCs are collected either through multiple aspirations from the iliac crests, or leukapheresis **[G]** following the administration of mobilizing agents **[G]** (**Fig. 3**) and collected material is enriched for CD34+ cells. Both procedures yield a mixture of non-engrafting cells and an heterogenous population of primitive and lineage-committed HSPCs that includes short-term repopulating haematopoietic progenitors contributing to early reconstitution and a very small fraction of long-term repopulating haematopoietic stem cells (HSCs) that take longer to restore haematopoiesis **(Fig. 1)**. The relative composition of HSCs and haematopoeitic progenitor cells (HPCs) in the collected material depends on the collection method, disease background, age and the mobilization procedure17-20. Indeed, the yield of CD34+ cells following bone marrow aspiration negatively correlates with donor weight and age21. Preliminary results of clinical trials for immunodeficiencies such as Wiskott-Aldrich Syndrome indicate that both mobilized peripheral blood and bone marrow sources engraft and lead to clinical benefit22, although in-depth comparative studies between the two sources are lacking. However, as leukapheresis provides more HSPCs than bone marrow harvest per donor and allows for faster hematopoietic reconstitution23 it is now the preferred procedure. G-CSF was the first mobilizing agent used for allogeneic transplantation; however, as some patients have a limited mobilization response to G-CSF23 it is usually given in combination with the CXCR4 antagonist Plerixafor in the context of gene therapy22,24,25,26,27. Interestingly, administration of Plerixafor alone has been shown to enrich for HSPCs with primitive and repopulating features — albeit with a lower yield than when used in combination with G-CSF19. Some studies have shown the feasibility of purifying primitive HSPCs28,29 for use as a starting population for ex vivo gene therapy; however, it is likely that primitive HSPCs will take longer than more committed progenitors for reconstitution and should therefore be combined with more committed progenitors for timely engraftment.

***[H2] Vector-based modification of HSPCs***

Following HSPC collection and enrichment, CD34+ cells are subject to gene transfer. Most gene transfer approaches to date have used viral vectors such as gammaretroviruses and lentiviruses to integrate a therapeutic gene into the genome of the recipient cell **(Fig. 4A)**. In the early ‘90s, Moloney murine leukemia virus (MoMLV)-derived gammaretroviral vectors were the first vectors employed for HSPC gene therapy for the treatment of immunodeficiencies including (ADA-SCID)30,31 and followed by HIV-derived, self-inactivating (SIN) lentiviral vectors32 in 2006 for X-ALD33 and SIN gammaretroviral vectors for X-SCID in 201034. Lentiviral vectors have become the most commonly-used vector for ex vivo HSPC gene therapy because of their good safety profile; lentiviral vectors preferentially integrate into gene bodies over promoters, reducing the potential for aberrant transcriptional activation (Fig. 4B). Further, in SIN vectors, the viral promoter located in the lentiviral vector LTR is inactivated upon integration into the genome, limiting transcriptional transactivation of cellular genes**.** Modifications in vector structure, including the deletion of promoter viral sequences in the LTRs and the splitting of viral protein genes across separate plasmids provided in trans during vector particle production have since contributed to a reduction in genotoxic risk associated with transcriptional transactivation and aided the production of replication-defective vector particles35**.** Although lentiviral vector-mediated gene therapy has an excellent clinical safety record, there remains a theoretical long-term risk of genotoxicity. In addition, the risks associated with high-dose chemotherapy — which is required in some cases for HSPC gene therapy — hamper the application of this approach for less severe conditions. There are also limitations that prevent the wider application of gene therapy approaches for genes that require physiological control of gene expression or gain-of-function mutations.

***[H2] Gene editing approaches***

Site-specific genome editing using programmable endonuclease platforms such as CRISPR–Cas9, transcription activator-like effector nucleases (TALENs) or zinc finger nucleases (ZFNs)36 can inactivate harmful alleles, disable transcriptional repressor expression or their binding sites, precisely correct mutations, or insert healthy gene copies into a genomic ‘safe harbour’ **(Fig 5)**. Precise gene correction may address potential issues associated with the semi-random integration of viral vectors, although it should be noted that so far no insertional mutagenesis has been reported with lentiviral vector-mediated gene therapy. Further, targeted gene editing gives the advantage of bringing the therapeutic gene under the control of endogenous regulatory elements, allowing for a physiological level of expression together with the potential of correcting diseases regardless of the type of mutation37,38. Recent studies showed proof-of-concept results in hemoglobinopathies39 and the immunodeficiencies SCID-X140,41,42 and WAS43.

DNA double-strand breaks induced by gene editing nucleases can be repaired through homology directed repair (HDR) if a donor homologous template is provided, or by error-prone non-homologous end joining (NHEJ). HDR can be exploited to correct disease-causing mutations, whereas NHEJ leads to gene inactivation, potentially correcting gain-of-function and dominant-negative mutations or inducing therapeutic knockout. Ex vivo gene editing of HSCs has been shown to result in efficient NHEJ44; however, HDR in long-term repopulating HSCs occurs at low levels40. HDR may not be as efficient in long-term HSCs as in progenitor cells because it mostly occurs in cell cycle phases (S/G2) incompatible with the HSC quiescent state and because of inefficient template uptake, which could limit the clinical translation of gene editing in diseases requiring a high frequency of gene-modified HSPCs such as lysosomal storage diseases. Chemical modification of single-strand guide RNA and innovations in the delivery systems of nucleases such as pre-assembled CRISPR-Cas9 RNPs and the use of donor templates based on AAV6 have increased the frequency of edited HSCs with promising results for future successful development and clinical application39,44-47. More recently,strategies to force cell cycle progression and to upregulate the expression of cellular components of the HDR machinery led to the successful increase of gene correction in up to 50% of in vivo repopulating HSCs46.

Proof of principle for the gene editing of HSPCs has been achieved in preclinical studies39,45,48,49, leading to two ongoing industry-sponsored clinical trials using ZFN and CRISPR-Cas9 technologies knocking-out *BCL11A* to address transfusion-dependent ß-thalassemia (TDT) and sickle cell disease (SCD), respectively (**Table 1**). *BCL11*A encodes a protein that represses the expression of HbF. Initial results from a patient with TDT and a patient with SCD showed good editing efficiency of HSPCs using CRISPR-Cas9 to disrupt *BCL11A*, increasing levels of total haemoglobin and fetal haemoglobin (HbF) over time in both patients, indicating successful engraftment of the edited cells50. SCD represents an ideal model for HDR gene editing approaches as it is caused by a single nucleotide mutation in a single gene. Studies using CRISPR-Cas9 to correct the SCD mutation — which is present in *HBB,* the gene encoding β-globin — showed HDR-mediated correction in isolated CD34+ cells was effective *in vivo*, but the frequency of corrected cells decreased after xenotransplantation to less than 10%, indicating differential targeting of progenitors versus repopulating HSCs could limit the clinical application of this approach45,51. Further strategies for addressing SCD have exploited NHEJ to abolish the expression of BCL11A. Disrupting either an erythroid-specific enhancer in the *BCL11A* gene52 or BCL11A binding sites in the promoters of the -globin genes *HGB1* and *HBG2*53,54 led to an elevation in HbF levels in vitro. Immunodeficient mice injected with CD34+ cells corrected by CRISPR–Cas9 showed a high frequency of edited HSCs and downregulation of *BCL11A* expression,and erythroid cells derived from edited engrafted cells showed correction of the SCD phenotype in vitro44. An alternative approach for targeting SCD has also been explored that mimics the condition of hereditary persistence of foetal haemoglobin (HPFH) through using TALEN and CRISPR-Cas9 to introduce deletions into the β-globin locus that interfere with gene regulation55,56,57,58**(Fig. 5)**. A proof-of-principle study using this strategy showed increased HbF expression and the correction of sickling in erythroid cells differentiated from patient CD34+ cells, and the persistence of edited cells after transplantation in immunodeficient mice58.

Gene editing technologies do have potential risks associated with off-target effects and DNA rearrangements such as chromosomal translocations and large deletions59, which are difficult to predict in preclinical models. In the case of HbF reactivation, the lack of a marker could make the origin of an increase in HbF difficult to determine as increased HbF is usually observed as a consequence of the transplantation procedure in general, especially in patients with SCD or β-thalassaemia60,61. Further advances in gene editing such as base editing and prime editing62-64 — which use a catalytically-impaired CRISPR/Cas9 carrying nickase activity but lacking DSB activity in association with a deaminase and reverse transcriptase, respectively — hold promise for efficient and possibly safer genome engineering, although most results have been obtained in cell lines and not in primary human cells.

***[H2] Cell culture and transduction***

In both vector-mediated gene transfer and gene editing approaches, the in vitro culture and manipulation of HSPCs can induce transcriptional responses and signaling events that may affect both primitive and gradually more committed cell populations65,66 and reduce the frequency of gene-corrected long-term repopulating HSCs. For this reason, a high dose of transplanted HSPCs is desirable both for fast haematopoietic recovery after conditioning and stable clinical outcome. Viral-mediated transduction of primitive HSCs requires pre-stimulation of the cells with activating cytokines such as stem cell factor (SCF), FMS-like tyrosine kinase 3 ligand (Flt3L) and thrombopoietin (TPO) and the use of high concentrations of vector capable of a high infectivity during ex vivo culture. The optimization of cell culture conditions such as culture duration and the choice or concentration of cytokines and transduction enhancers can enable the highest transduction efficiency or gene editing with the minimum loss of primitive HSC function. Indeed, culture time (usually between 24–48 hours) negatively correlates with the maintenance of repopulation capacity, as cytokine stimulation favours the expansion of more committed progenitors at the expense of repopulating HSCs28,67. Reliable assays to define transduction efficiency in long-term repopulating HSCs — one of the key factors for the prediction of favorable outcome— are still lacking.

The potency specifications for a gene therapy drug product currently rely on surrogate parameters to measure potency. These parameters include the average number of vector copies per genome detected in HSPCs a few days after vector exposure, the proportion of successfully transduced clonogenic progenitors, transgene expression and corrected function.There are currently no available markers for the evaluation of repopulating HSCs following in vitro culture; consequently, multiple clinical trials have shown a lower rate of genetic modification in engrafted cells than those tested in vitro24-26,68. However, this is not the case for clinical trials investigating immunodeficiencies13,14,22 and Fanconi anaemia27, in which corrected cells are endowed with a proliferative advantage. The efficiency of transduction can be limited and variable among individuals, likely due to differences in the quiescence state of the isolated HSPC population and the expression of antiviral restriction factors by HSPCs69 that act at different steps of the transduction pathway7,70. Recent advances using transduction enhancers ex vivo could improve transduction efficiency; for example, compounds such as poloxamers71,72, rapamycin73, cyclosporine A, cyclosporine H70 and prostaglandin E2 (PGE2)28,74 can enhance targeting of the vectors to HSPCs by enhancing vector particle attachment or entry, or acting on post-entry phases allowing for a higher level of integration. However, long-term clinical trials are needed to assess the efficacy of these drugs for improving the efficiency of HSPC engraftment.

***[H2] Patient conditioning***

Conditioning regimens using chemotherapeutic or immunosuppressant drugs aim to deplete a patient’s endogenous HSPC population in order to clear a niche for engraftment of corrected cells while mitigating toxicity. Conditioning can range from reduced intensity to myeloablative conditioning, **[G]** depending on the disease, the required level of transgene expression and the engraftment level required to reach the therapeutic threshold75.

In autologous HSPC gene therapy, reduced intensity conditioning allows the establishment of a stable mixed chimerism of uncorrected and corrected cells, which is possible due to the absence of rejection and graft versus host effects.This chimerism is favored when corrected cells are endowed with an in vivo proliferative advantage, for example, in the case of lymphoid cells in immunodeficiencies13,14,22 and corrected HSPCs in Fanconi anaemia76. However, reduced intensity conditioning strategies are usually insufficient for diseases such as lysosomal storage disorders or haemoglobinopathies24,25,68,77 that require a high degree of engraftment. For example, myeloablative conditioning may be required for HSC gene therapy of β-thalassemia in order to secure sufficient space in the bone marrow and allow the engraftment of an adequate dose of genetically engineered HSCs for differentiation into corrected red blood cells. For neurometabolic disorders, conditioning regimens based on alkylating agents are preferred due to their ability to cross the blood–brain barrier, deplete resident microglia cells and favor the local migration of corrected cells 78. Toxicity associated with conditioning can be mitigated using pharmacokinetic techniques; for example, the toxicity of the alkylating agent busulfan is currently mitigated by serial evaluations of concentrations to determine the area under the curve (AUC)75, followed by dose adjustment.

Infertility is a major risk of myeloablative chemotherapy that can be addressed by gonadal cryopreservation in children79. Targeted and non-genotoxic methods for myeloablation as alternatives to conventional chemotherapy have recently been developed based on antibodies that recognize HSC surface markers. Studies in mice and non-human primates demonstrated the efficacy of anti-CD117 (c-Kit) and anti-CD45 antibodies for the depletion of resident HSCs80,81. Recently, a phase I clinical study assessing the safety and tolerability of allogeneic transplantation in patients with SCID treated with an anti-CD117 antibody showed promising initial results with successful engraftment of donor cells82. If proven to be safe and efficacious, these therapeutic antibodies could be used for conditions in which the risk of chemotherapy is deemed too high, or where the pre-existent inflammatory status of the bone marrow is further exacerbated by the effect of conventional conditioning regimens.

The bone marrow microenvironment plays a key role in facilitating the engraftment and expansion of genetically-corrected cells. Recent studies found a defective bone marrow environment in β-thalassemia was associated with impaired HSC function that could negatively affect engraftment83,84. A patient’s age may also have an impact on bone marrow status and the quality of HSPCs; a recent study showed younger patients were associated with better outcomes following HSPC gene therapy for TBT25.

**[H2] Engraftment of modified HSPCs**

Vector-transduced haemopoietic cells can be detected in the blood by RT-PCR as early as one week after infusion. Corrected granulocytes and monocytes are first detected, followed by B cells and NK cells and eventually T cells, which must first reach maturation in the thymus13,85. The majority of patients treated with lentiviral-mediated HSPC gene therapy display stable long-term engraftment of transduced cells and polyclonal haematopoietic reconstitution up to 8 years post-treatment8,22, and patients with ADA-SCID treated with gammaretroviral vectors show engraftment up to 15 years post-treatment86. In both cases, engraftment results in persistent expression of the transgene driven by the integrated vector, as assessed by immunological and/or biochemical monitoring. Correction of the disease phenotype has been reported in most treated patients, the degree of which usually correlates with levels of engraftment and transgene expression in vivo. The extent of correction of repopulating HSPCs has been variable among trials, ranging from 0.1% to 80%77 of gene-modified HSPCs. The highest and most consistent levels of engraftment for various lineages have been observed in studies using HSPCs corrected using lentiviral vectors and a myeloablative patient conditioning strategy7. Engraftment failures have been associated with insufficient doses of HSPCs or insufficient transduction efficiency, a lack of patient conditioning or the presence of concomitant diseases such as underlying infections75,75,87,88,89,90.

The human bone marrow niche comprises several non-haematopoietic cells, including mesenchymal stromal cells (MSCs), osteoblasts, adipocytes, endothelial cells and neural cells that offer physical support and secretion of soluble factors to HSPCs and regulate their homeostasis91. MSCs are localized in the endosteum and around the sinusoidal vessels92 and can be classified into functionally distinct subpopulations, identified based on the expression of CD146 (Melanoma cell adhesion molecule, or MCAM), CD271 (NGF receptor, or NGFR), STRO-1 and SSEA-4 markers93,94. Humanized niche models represent a powerful tool for dissecting the haematopoietic supportive function of distinct MSCs subpopulations in vivo within a microenvironment that mimics the human situation95,96. MSCs have been used in the clinical setting to expand ex vivoHSPCs97 and a recent pilot study described the infusion of MSCs to facilitate HSPC engraftment97,98. These studies suggest MSCs could be employed in the context of HSPC gene therapy.

**[H2] Vector integration and clonal tracking**

Viral vectors retain the ability to integrate into the cell genome semi-randomly, with some regions preferred to others depending on the class of retrovirus vector used. Lentiviral vectors preferentially insert into actively transcribed gene bodies, whereas gammaretroviral vectors preferentially integrate close to transcription start sites and active regulatory elements, specifically99,100,101,102,103,104. Semi-random vector integration could potentially induce changes in gene structure or expression that may provide a selective clonal growth advantage or result in uncontrolled proliferation105. The first vector integration studies in patients with ADA-SCID treated with gammaretroviral vectors showed the existence of insertion sites shared among multiple haematopoietic lineages, indicating bona fide engrafted, transduced HSPCs102 106,107, although at the time these studies were carried out the efficiency of insertion site detection was still low, and most clones were derived from lymphoid lineages with a selective advantage106,107. Studies in immune deficient patients showed that genes implicated in cancer are frequent insertion sites for gammaretroviral vectors; this may be as a result ofpreferential targeting for these integrations at the time of transduction102 and/or that cells with these integrations are observed at a higher frequency due to clonal expansion of these cells after cell infusion108,109. Linear-amplification mediated PCR (LAM**-**PCR) amplification and other methods that avoid restriction enzymes105, coupled with next-generation sequencing of virus–host DNA junction sequences, has since been performed routinely in clinical trials to monitor insertion site profiles and clonal fluctuation of the engrafted population110,111.

Oligoclonality **[G]** and enrichment of insertions near proto-oncogenes has been observed in patients treated with gammaretroviral vectors. Insertion site analyses have revealed the presence of integration sites near proto-oncogenes such as *LMO2* in leukaemic clones that developed in SCID-X1, Wiskott–Aldrich syndrome and CGD trials15,108,112. However, continuous insertion profiling was not able to detect the onset of leukaemia in patients who went on to develop the disease following HSPC gene therapy until clinical symptoms had become apparent111. Thus, the clinical application of insertion site analyses is currently limited to investigational studies and analyses of abnormal clinical findings but not real-time monitoring.

A polyclonal pattern of vector integration has been observed in the majority of patients at long follow-up times, with a balanced proportion of different clones.So far, no dominant clones enriched for vector insertion within oncogenes have been detected in lentiviral vector trials**,** with the exception of a single patient in a β-thalassaemia study113. The patient was found to have a dominant clone harbouring an integration in the *HMGA2* gene, causing deregulation of *HMGA2* expression that was not associated with adverse effects. The risk of genotoxicity for lentiviral insertions using a safe vector backbone and an adequate dose of HSC is therefore predicted to be low68,99,114. Risk will further be reduced through safety monitoring of patients treated with lentivirus-modified HSPCs for at least 15 years115.

Sequencing data generated as part of integration site analysis can be used to establish relationships between human haematopoietic lineages during distinct phases of haematopoietic reconstitution, as well as estimate the number of long-term HSCs that participate in haematopoietic cell production after engraftment116,117. It has been estimated in subjects treated with HSPC gene therapy that approximately 1/105 to 1/106 of the infused HSPC cell population contributes to long-term haematopoiesis68,114. Recent studies monitoring the kinetics of blood cell production from individual HSPCs suggest that distinct subtypes contribute to early and late post-transplantation phases differently. In particular, these studies showed that multi-potent progenitors activate soon after transplant and dominate initial haematopoietic output, whereas long-term repopulating HSPCs become predominant 1–2 years after transplantation, once haematopoiesis reaches a steady state116,118. However, it should be noted that these studies are based on data derived from gene therapy trials for PIDs and could be biased towards reconstitution of the lymphoid compartment.In cases where gene editing is used in HSPC gene therapy, studying the safety and dynamics of the corrected cell population could prove challenging owing to the lack of a distinctive element identifying each clone, especially when high fidelity homology repair (HDR) is performed. Clonality studies may be useful in the case of non-homologous end joining (NHEJ) editing, since the DNA breaks produces insertions and deletions (indels) of different length that can be sequenced by next generation sequencing techniques.

**[H1] Applications for HSPC gene therapy**

**[H2] Primary immunodeficiencies**

Primary immunodeficiencies (PIDs) are a heterogeneous group of rare heritable disorders that result in an underdeveloped and/or functionally compromised immune system119. Patients with severe PIDs such as forms of severe combined immunodeficiency (SCID), combined immunodeficiency (CID) or severe myeloid cell disorders experience increase morbidity and mortality and display diverse clinical phenotypes120. HSCT using HSPCs from an HLA-matched donor can confer a lifelong ‘cure’, with a success rate of more than 90%121,122,123; however, the limited availability of HLA-matched donors, particularly in certain populations and non-western geographies, results in the necessity to use unmatched donors, which increases treatment-related risk due to GVHD, toxicity and infections, the risks of whichdepend on age, co-morbidities and genotype123. Less severe PIDs than SCID and CID can still have a major impact on quality of life, and HSCT may be especially beneficial for conditions that require lifelong treatment with drugs and supportive therapies, which for many immunodeficiencies are only partially effective. Such treatments can also be limited by their availability and cost, particularly in the case of immunoglobulin replacement in antibody-deficiency syndromes or recombinant enzyme replacement therapies.

*[H3] X-linked severe combined immunodeficiency*

SCIDs are characterized by a depletion or functional deficiency of T lymphocytes and often associated with B lymphocyte and/or NK cell deficiencies120 As a result, these conditions are associated with a high mortality in early life owing to severe infection. The success of HSCT gene therapy in treating many of these conditions is likely due to both a growth and survival advantage for gene-corrected cells and the inability of patients with SCID to reject allogeneic grafts. Because of these factors, successful engraftment of graft lymphocytes can occur without the need for cytoreductive conditioning124. The capacity for PID patients to accommodate donor lymphocytes, predominantly T cells, is unsurprising considering patients with PID can acquire somatic mosaicism and a milder or ‘atypical’ phenotype through spontaneous genetic reversions and second-site mutations125.

SCID-X1 is caused by a deficiency of the cytokine receptor common subunit γ (also known as IL-2RG) — a critical component of multiple cytokine receptors including the IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors — which results in the absence of T cells and NK cells, and the presence of functionally deficient B cells126. Early trials conducted using conventional gammaretroviral vectors with intact LTRs demonstrated rapid reconstitution of T-cell immunity as a result of gene-corrected HSPC-mediated initiation of thymopoiesis in 17 of 20 patients treated34,87 (**Fig. 2**). Humoral immunity was only partially restored, as the absence of cytoreductive conditioning and sustained HSC engraftment in these studies meant the resident B lymphocyte lineages remained uncorrected. Somewhat surprisingly, active thymopoiesis seemed to be maintained for many years after treatment126,127, suggesting durable thymic engraftment of a very long-lived lymphoid or T-cell progenitor even in the absence of corrected long-term repopulating HSPCs. However, these promising clinical data were tempered by the development of T-lymphoblastic leukaemia in over 25% of patients as a result of insertional mutagenesis15,112. The combination of semi-random vector genome integrations and potent enhancer sequences within the pro-viral LTR caused dysregulated expression of known proto-oncogenes including *LMO2*, which has since been recognized as a major contributor to leukaemogenesis128. This, in combination with additional clonal genetic alterations — likely occurring stochastically or associated with proliferative stress — precipitated clinically-manifesting leukaemia15. The occurrence of similar pathologies in other trials not related to SCID-X1108 using gammaretroviral vectors indicated the gene transfer technology specifically was inducing leukaemia. To address these issues, alternative vector platforms based on LTR enhancer-deleted gammaretroviruses34 and more recently lentiviruses129 have been deployed in a number of clinical trials for SCID-X1, with sustained immune reconstitution and clinical improvement and no evidence for clonal dysregulation or leukaemogenesis over more than 6 years of follow-up

Recently, the use of low-intensity cytoreductive conditioning has facilitated successful long-term engraftment of gene-corrected HSCs and functional restoration of B lymphopoiesis as well as thymopoiesis in patients with SCID-X1130. Older patients have also benefited from gene therapy with at least partial T cell recovery following failed allogeneic procedures, even though thymopoiesis has been dormant for many years129. This observation suggests that even at late stages, thymopoiesis is a retrievable developmental programme as long as there is a supply of gene-corrected T cell progenitors.

*[H3] Adenosine deaminase-deficient severe combined immunodeficiency*

ADA-SCID is an autosomal recessive metabolic disease caused by mutations in the gene encoding adenosine deaminase(ADA), which result in the accumulation of toxic metabolites such as deoxyadenosine that compromise lymphoid development. Enzyme replacement therapy through weekly administration of PEGylated bovine or recombinant adenosine deaminasecan ameliorate the build-up of these metabolites in the blood and allows variable restoration of lymphopoiesis, albeit at a high financial cost8. Early attempts at gene therapy targeting residual mature T lymphocytes had minimal clinical benefit as patients were maintained on enzyme replacement therapy (ERT)9,10. However, the use of HSCs and the introduction of a low-intensity conditioning regimen to facilitate their engraftment and multi-lineage reconstitution produced remarkable clinical responses14,88,131,132 **(Fig. 2)**. Strimvelis, a gene therapy based on the introduction of ADA genes into HSCs using a gammaretroviral vector, was the first ex vivo gene therapy to be licensed in 2016133. Despite the presence of intact gammaretroviral LTR enhancer sequences and evidence suggesting clonal dysregulation occurs at the molecular level following treatment134, no instances of clinically-manifesting mutagenesis have occurred 86,131,135. A leukaemic clone could be selected against due to its high requirements for the products of purine metabolism, which may be present at low levels in ADA-deficient patients136. Owing to the metabolic nature of the transgene and ADA being ubiquitously expressed, the ADA-defective bone marrow–thymus microenvironment could restrict or ‘protect’ haematopoietic or thymopoietic cells from proliferative stress by partially compromising cell division and differentiation; however, this process is not fully understood. It should be noted that patients receiving enzyme replacement alone can also develop lymphoproliferation137.

*[H3] Other primary immunodeficiencies*

Many other PIDs that are currently treatable using allogeneic HSCT are viable targets for an autologous gene therapy approach. Wiskott–Aldrich syndrome is a complex, multi-lineage PID caused by mutations in the *WAS* gene that result in haematopoietic cell cytoskeletal dysfunction, and is further complicated by thrombocytopenia **[G]**138. Use of a conventional gammaretroviral vector to treat this condition resulted in clinical benefit but with an unacceptable degree of leukaemic toxicity108,136 ; however, a lentiviral platform incorporating a proximal segment of the *WAS gene* promoter has recently demonstrated sustained immunological correction and abrogation of bleeding tendency in over 30 children and adults severely affected by Wiskott-Aldrich syndrome without clonal dysregulation89,114,22,139. Attempts at gammaretroviral correction of CGD — a group of conditions characterized by deficiencies the NADPH oxidase system — were limited by lack of efficacy due to low engraftment, mutagenesis and transgene silencing as a result of LTR promoter methylation109. Haematopoietic proliferative stress and a decrease in the repopulating activity of HSCs have been observed in the CGD mouse model, suggesting that the chronic inflammation observed in patients with CGD could negatively affect the outcome of gene therapy unless adequately controlled140.

Recent developments in the treatment of CGD have successfully restored long-term biochemical and immunological function in patients with severe CGD, allowing withdrawal of regular medications90. These developments include the refinement of a myeloablative conditioning regimen — necessary because myeloid cells, which are the predominant cell type affected by CGD, require continual lifetime renewal from HSCs — and the use of a lentiviral vector incorporating a regulatory element designed to avoid mutagenesis in HSPCs and mediate more physiological transgene expression patterns. This regulatory element is also being utilized in a clinical study for leukocyte adhesion deficiency (LAD) type I, for which correction of the myeloid lineage is the principal goal. More recently, lentiviral vector platforms have been developed for the treatment of other forms of SCID, including those caused by deficiencies in *DCLRE1C* 141 and *RAG*8,142 (Table 1).

**[H2] Erythrocyte disorders**

Genetic diseases that affect red blood cells and are treatable using HSPC gene therapy include β-thalassaemia, SCD and pyruvate kinase deficiency (PKD). In SCD and β-thalassaemia, both of which are caused by mutations in *HBB* — the gene that encodes β-globin — gene therapy is particularly challenging because the inclusion of large-scale *HBB* genomic sequences and locus control region elements is a limiting factor in the design and manufacture of high-titre vectors143. From a safety point of view, the use of erythroid-specific regulatory sequences restricts the genotoxic risk of gene transactivation to erythroid precursors committed to enucleation and with a limited half-life.

*[H3] β-thalassaemia*

In β-thalassaemia, mutations in *HBB* — the gene encoding the β-globin chain of haemoglobin — result in an imbalance between α-globin and β-globin chains that is toxic to erythroid precursors. β-thalassaemia major is a particularly severe form of β-thalassaemia associated with chronic and severe anaemia, caused by homozygous inactivating mutations in the β-globin gene (represented as βo/βo) and currently requires lifelong monthly blood transfusions and iron chelation treatment **[G]**. Allogeneic HSCT has been used to cure β-thalassaemia, but is only available to the minority of patients who have a compatible donor144.

HSPC gene therapy using erythroid-specific globin-expressing lentiviral vectors was the first strategy targetingβ-thalassaemia successfully translated to clinical trials in 2006 (Table 1). The safety and efficacy of the BB305 lentiviral vector, which encodes a β-globin transgene (βT87Q globin) with anti-sickling properties, were reported in phase I and II trials of patients with varying severities of β-thalassaemia24. Clinical outcome was dependent on genotype, with 80% of patients with non-β0/β0 genotypes and 38% with β0/β0 genotypes achieving transfusion independence at the 2 year follow-up. The remaining patients exhibited varying levels of transfusion reduction. On the basis of these results, in 2019 the EMA gave conditional marketing authorization to ZyntegloTM for patients with TDT with non-β0/β0 genotypes **[Au:OK?]**. Optimization of the transduction protocol led to the start of two phase III clinical trials that are current ongoing (Table 1, NCT02906202 and NCT03207009). In the TIGET-BTHAL clinical trial (Table 1, NCT02453477), nine patients with β0/β0 genotypes, including six minors, were treated using intra-bone administration of GLOBE lentiviral vector-transduced HSPCs. In the 1 year follow-up, the primary endpoints of transfusion reduction and safety were achieved in all patients**,** with four patients achieving transfusion independence. Patients with clinical benefit showed robust and stable engraftment of genetically-modified cells in all lineages, including bone marrow erythroid cells25. Updated results showed a better outcome in minors than adult patients145. Studies on the bone marrow microenvironment in β-thalassaemia and its potential impact on HSPC function83,84 suggest the bone marrow microenvironment could impact clinical outcome; for example, impairment of stromal niche cells caused by disease-related secondary effects, such as ineffective erythropoiesis, iron overload or bone defects, has been reported in patients83 and in thalassaemic mice with defective HSPC function in the latter caused by altered niche–HSPC crosstalk84.

*[H3] Sickle-cell disease*

The bone marrow microenvironment is a key factor for HSC quality in SCD, which is linked to a chronic inflammatory environment, abnormal bone marrow vascular network and stress erythropoiesis **[G]**146,147. SCD is caused by a single-nucleotide mutation in HBB, resulting in the production of the toxic haemoglobin variant HbS, which polymerizes into long fibres that deform red blood cells into a sickled shape. Gene therapy strategies for SCD either use transgenes encoding foetal globin or β-globins with anti-sickling activity such as βT87Q, βAS3; reactivate HbF production by generating HPFH mutations, or suppress the biological action of BCL11A, a major repressor of globin genes148,149.

The first HSPC gene therapy strategy used to treat a patient with SCD employed a BB305 lentiviral vector and bone-marrow derived HSPCs to deliver a transgene encoding anti-sickling β-globin. This approach achieved a therapeutic level of protein in erythrocytes corresponding to 50% of β-like globin chains, with successful correction of clinical symptoms150. Similar results were reported in one of two other treated patients. However, unsatisfactory results were obtained in seven initial patients treated in another trial150. Using Plerixafor-mobilized HSPCs instead of bone marrow-derived HSPCs and increasing the transduction level with an culture optimized protocol151 has since improved the clinical outcome, with recent results showing sustained expression of transgenic anti-sickling β-globin and decreased SCD manifestations152. Two further phase I and II lentiviral vector-based clinical trials for SCD are currently ongoing; in one trial, the patients are subject to reduced-intensity conditioning and transplanted with HSC expressing the -globin gene153, whereas in the other a lentiviral vector containing a β-globin transgene carrying three anti-sickling mutations (βAS3) was used. Early results have shown the treatment is safe and shows some benefit although long-term follow up will be needed to gauge efficacy. Finally, a different approach exploited lentiviral vector-mediated erythroid-restricted expression of a small hairpin RNA targeting *BCL11A* to reduce the repressive action of BCL11A on HBG genes and consequently upregulate HbF levels154. A phase I clinical trial using this technology started in 2018 for patients with SCD (**Table 1**), with promising initial results showing HbF reactivation155. Long-term follow-up will be needed to confirm the origins of the increased HbF level and amelioration of clinical symptoms.

*[H3] Pyruvate kinase deficiency*

PKD is caused by a mutation in *PKLR* and is associated with haemolytic anaemia. Patients with severe PKD often require chronic blood transfusions and treatment with iron chelators. Lentivirus-mediated HSPC gene therapy transferring *PKLR* cDNA has shown proof of efficacy and safety in a mouse disease model156, and a phase I/II clinical trial has recently been approved.

**[H2] Bone marrow failure syndromes**

Fanconi anaemia is a congenital, autosomal recessive DNA repair disorder associated with developmental abnormalities, bone marrow failure and a predisposition to cancers. A number of genes involved in DNA replication and repair have been implicated in Fanconi anaemia, including *FANCA* and *FANCB.* In a recent clinical study, patients with Fanconi anaemia caused by *FANCA* mutations received lentivirus-mediated HSPC gene therapy aiming to transfer an intact copy of the *FANCA* gene27. Patients were not conditioned in order to avoid drug toxicity. The study showed a gradual increase in corrected bone marrow CD34+ cells, with the proportion of corrected cells ranging from 7% to 43% at 18–30 months follow-up. Lentiviral vector-mediated gene therapy conferred a selective proliferative advantage to transduced HSCs and corrected blood cells by inducing resistance to DNA cross-linking agents as assessed in vitro. As expected, patients with the lowest marking levels in integration site analysis had the most limited number of repopulating clones fluctuating over time, and none of the treated patients have shown signs of genotoxicity so far. This approach was able to halt the progression of bone marrow failure in patients with the highest levels of gene-corrected cells. Recently, preclinical studies using CRISPR-Cas9-driven NHEJ to create compensatory mutations restoring the coding frame in HSPCs from Fanconi anaemia patients showed a marked proliferative advantage for edited HPSCs in vitro and in xenotransplanted mice, with efficient correction of the Fanconi anaemia disease phenotype157.

**[H1] HSPCs as delivery vehicles**

**[H2] Inherited neurometabolic disorders**

Mutations in lysosomal or peroxisomal enzymes can cause the accumulation of toxic substrates in multiple organs, including the CNS; buildup of toxic substrates in the CNS specifically can lead to severe neurological damage158. Systemic administration of ERT is approved for some metabolic disorders but its application for neurological disorders is hampered by the inability of proteins to cross the blood–brain barrier. Further, skeletal deformities associated with lysosomal storage diseases remain difficult to treat using ERT owing to the insufficient biodistribution of ERT into cartilage and bone158. These issues could be addressed using HSPC gene therapy as HSPCs and blood cells of the myeloid lineage are able to penetrate into the CNS and replace the resident populations of tissue macrophages and microglia-like cells159,160. Indeed, mouse models of transplantation using chemotherapy-mediated ablation of brain-resident myeloid cells have shown effective replacement of these cell populations with donor-derived HSPCs78, and damage to microglia has been implicated in the pathogenesis of both peroxisomal and lysosomal storage diseases161. In the latter case, lysosomal enzymes secreted by corrected CNS-migrated, HSPC-derived myeloid cells could enable the correction of adjacent cell types — for example, oligodendrocytes, neurons and astrocytes — owing to the capacity of these cells to uptake enzymes through the mannose-6-P receptor present on their cell surface. Such enzymes include arylsulfatase A (ARSA) and alpha-L-iduronidase (IDUA), which are deficient in metachromatic leukodystrophy and mucopolysaccharidosis type I, respectively. HSPC gene therapy could also facilitate the replacement of functionally-defective myeloid cells, including macrophages and microglia, restoring scavenging functions and contributing to ameliorate inflammation and oxidative stress in the brain161,162.

Allogeneic HSCT is currently used as a therapeutic option in early forms of the peroxisomal disease X-linked adrenoleukodystrophy (X-ALD)163, and in selected lysosomal storage disorders, such as mucopolysaccharidosis type I (MPS I). However, some patients still experience significant neurological disease burden even after successful transplantation164,165. Allogeneic HSCT is currently ineffective in diseases such as MPS IIIA and in metachromatic leukodystrophy (MLD) especially in their early-onset forms158. This may be because of insufficient cross-correction of the metabolic defect in non-hematopoietic cells in the short time available to control rapid disease progression. Genetic modification of HSPCs designed to overexpress therapeutic proteins could therefore increase therapeutic protein levels in the blood and multiple tissues including the CNS and bone and provide enhanced cross-correction capacity, compensating for the presence of residual, uncorrected host cells (**Fig. 6**) Individual neurometabolic disorders are discussed below.

*[H3] X-linked adrenoleukodystrophy*

X-linked adrenoleukodystrophy (X-ALD) is a severe demyelinating disease caused by defects in the degradation of very long-chain fatty acids induced by a deficiency in ALD protein (encoded by *ABCD1*). The cerebral form of X-ALD is characterized by learning and behavioral problems starting at a median age of 7 years, followed by rapid neurological deterioration. Brain inflammation with infiltration of monocytes and microglia loss are hallmarks of the disease. The beneficial effects of transplantation are thought to be mediated by donor replacement of defective myeloid cells26,161.

The first human gene therapy study with lentiviral vector-transduced HSPCs was performed in two patients with cerebral X-ALD33 (**Fig. 2**). Gene therapy resulted in *ABCD1* transgene expression in 19% of CD14+ myeloid cells, metabolic correction and clinical stabilization at 12–16 months after treatment. A subsequent trial saw similar results in 15 of 17 patients with X-ALD followed up at a median time of 29.4 months after gene therapy, who were free of major functional disabilities and had attenuated progression of brain lesions26. Peroxisomal enzymes are not secreted and the mechanism of disease amelioration for peroxisomal storage diseases is still not well understood, although it has been hypothesized that corrected cells can restore the metabolism of non-functional neuronal cells through direct intercellular contact166.

*[H3] Metachromatic leukodystrophy*

Metachromatic leukodystrophy (MLD) is a severe lysosomal storage disorder caused by mutations in *ARSA*, a gene encoding arylsulfatase A (ARSA). ARSA deficiency results in the accumulation of sulfatides in the CNS and peripheral nervous system, leading to progressive demyelination and neurodegeneration. The first clinical trial based on HSPCs transduced with a lentiviral vector encoding *ARSA* showed safety and efficacy in eight out of nine MLD patients treated at a pre-symptomatic or very early-symptomatic stage68. Treatment restored ARSA activity in patient circulating haematopoietic cells and in the cerebrospinal fluid to normal or above-normal levels — indicating local production by corrected HSPC-derived cells — and either prevented disease onset or progression. In contrast to untreated patients and their siblings, most treated patients displayed continuous motor and cognitive development77 at a median follow up of 3 years; these results have since been confirmed in a larger cohort of patients up to 7.5 years of follow up167. Marketing authorization for this gene therapy based medicinal product (Libmeldy) is currently under evaluation in the EU. This study represents a key proof of principle indicating that HSPC gene therapy could be used as a therapeutic strategy for lysosomal storage disorders.

*[H3] Mucopolysaccharidosis*

MPS is a group of rare heterogenous diseases caused by deficiencies in enzymes involved in the breakdown of glycosaminoglycans (GAG). These diseases manifest with somatic and neurological symptoms, depending on the type of accumulating GAG168.People with the severe form of MPS type I (Hurler syndrome), which is caused by a deficiency in the enzyme iduronidase, usually develop skeletal abnormalities, hepatosplenomegaly, specific facial features, visual, heart and respiratory problems, and developmental delay, becoming severely intellectually disabled. The physical features of MPS type III patients (Sanfilippo syndrome) are less pronounced than those of with MPSI and these children develop neurodegeneration with impaired cognition and behavioral and sleeping problems. Proof-of-concept studies in mouse models of MPSI and MPSIIIA — which is caused by mutations in the gene encoding N-sulfoglucosamine sulfohydrolase gene — treated with lentiviral vector-modified HSPC gene therapy have shown increased efficiency of this approach over allogeneic HSCT169,170. Clinical trials aimed at correcting the neurological phenotype and systemic features of MPSI and MPSIIIA have recently started (Table 1). A preliminary communication on the MPSI gene therapy study shows supraphysiological blood IDUA activity, rapid reduction of GAG and early signs of clinical improvement171.

*[H3] Other gene therapy strategies*

Clinical trials based on lentivirus-transduced HSPCs are ongoing for the lysosomal diseases cystinosis and Fabry disease (see Table 1) and are in the preclinical phase for MPS II, MPSIIIB, and Pompe disease. It should be noted that the benefits of ex vivo HSPC gene therapy might be limited in symptomatic patients and patients with rapidly progressive disease variants because of the inherent delay in the enzymatic reconstitution of CNS tissues by HSPC-derived myeloid cells. Approaches based on direct intra-brain delivery of adeno-associated virus (AAV) or lentiviral vectors encoding the gene encoding for the defective enzyme172, intravenous injection of AAV vectors capable of targeting the brain, such as AAV9173 or the direct injection of gene modified haematopoietic progenitors174 might provide more timely enzyme reconstitution. Universal newborn screening for early detection of these diseases is under development or already being implemented as part of established metabolic screening programmes and could allow for prompt treatment before onset of symptoms175.

**[H2] Acquired diseases**

HSPCs could be exploited to deliver therapeutic molecules systemically or to affected tissues for the treatment of acquired diseases such as cancers, acquired immune disease, chronic infections or neurodegenerative disorders such as multiple sclerosis. HSPCs could be engineered to produce cytokines that induce resistance to specific microorganisms or express surface receptors that modulate the immune response; for example, when expressed on HSPCs, the immune checkpoint molecule PD-L1 was shown to inhibit autoimmune responses and reverted diabetes type 1 diabetes in an experimental model176. In addition, HSPC could produce molecules blocking tumor cell growth or facilitating immune recognition of cancer. Local release of therapeutic molecules delivered by HSPC could achieve sustained expression in target tissue while reducing systemic toxicity and the risk of adverse events. This was shown recently in preclinical models in which macrophage-infiltrating tumours derived from transduced HSPCs selectively expressing IFNα induced an immunostimulatory program in the tumour microenvironment, as shown by transcriptome analyses. This favored T cell priming and effector functions towards multiple tumor antigens, leading to inhibition of leukaemia growth177.

**[H1] Conclusions and future perspectives**

HSPC gene therapy offers the prospect of major clinical improvement and even cure for a large number of inherited immunohaematological and metabolic diseases. As autologous HSPC gene therapy reaches the clinic, there are significant financial and logistical challenges that must be addressed before its use on a global scale. Current strategies are based on centralized manufacturing facilities and limited number of treatment centers expert in the disease and gene therapy, whereas fully automated transduction could modify this model by increasing local manufacturing and widespread diffusion.

The development of stable producer cell lines and reagents capable of enhancing viral attachment (such as LentiBOOSTTM), suppressing natural intracellular viral restriction pathways (such as Cyclosporin H), and promoting transduction while shortening *ex vivo* culture (such as PGE2), are under intense investigation, with some already in clinical use151,171. Clonal tracking studies will be important to evaluate whether updated protocols preserve both primitive and committed progenitors in the drug product and favour rapid haematopoietic reconstitution, while simultaneously preventing rapid exhaustion of primitive HSCs and achieving long-term graft maintenance. Additionally, cryopreservation of the drug product or close automation of cell processing and transduction could allow greater standardization in terms of quality and dosing.

For most HSCT applications, the reliance on alkylating agents for patient conditioning is associated with short-term and long-term toxicities. The development of antibody-based conditioning regimens targeting molecules expressed on host haematopoietic cells such as CD117 (c-Kit) or CD45 could replace alkylating agents or be used in conjunction with them to allow for a reduction in alkylating agent dose.

Recent preclinical studies have shown a potential alternative to ex vivo gene transfer through transducing HSPCs after direct intravenous in vivo administration of viral vectors, albeit at low efficiency.178

In some cases, the physiological regulation of transgene expression is desirable to achieve a clinical effect and to avoid unwanted, transgene-related toxicity. Although incorporation of sophisticated regulatory elements into gene-addition vectors may realize these criteria, locus-specific gene editing could harness natural gene regulatory mechanisms. However, the use of synthetic minigenes as ‘universal’ repair templates for homologous recombination will not necessarily recapitulate physiological gene expression and may require sophisticated design. Improvements in gene editing efficiency, particularly in repair accuracy, will likely translate into an increasing number of clinical applications using gene edited HSPCs. Although in principle gene editing should be safer than vector-based gene addition approaches as it should avoid issues with off-target DNA changes caused by semi-random vector integration, the clinical safety of gene editing in HSPC gene therapy has yet to be proven179. The development of high-fidelity Cas9 nucleases could overcome any residual off target changes, although DNA double-strand breaks can also cause genome rearrangements such as deletions, inversions and translocations59. Base editing134 and prime editing62,63 hold promise for more safe and efficient genome engineering. Nevertheless, significant improvements in clinical-scale manufacturing and a better understanding of off-target and unexpected on-target effects are needed before implementation of gene editing in gene therapy. In the landscape of genome editing, preclinical studies and regulatory guidelines should be driven by previous experience from vector-based gene therapy, which has paved the way for the clinical translation of advanced cellular therapies. Assays and protocols must be adapted to better evaluate potential genotoxicity caused by gene editing.

Most HSC gene therapies to date have targeted defined inherited diseases. As technology improves, and our understanding of the durability and safety of HSC gene therapy increases, opportunities may arise to target other disease settings. For example, acquired neurodegenerative conditions could benefit from sustained delivery of therapeutic molecules to the brain through HSPC-derived microglia. Chronic infectious diseases and cancer could also be ameliorated by systemic delivery of therapeutics, or through elimination of haematopoietic disease reservoirs as in the case of HIV180. The genetic modification of T lymphocytes with chimeric antigen receptor (CAR), for example, represents an innovative approach to treat various forms of haematological cancer181. The use of HSPC gene therapy is likely to continue to grow rapidly and address an increasing range of immunohaematological and neurometabolic diseases.

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**Author contributions**

All authors researched the literature, provided substantial contributions to discussions of the content, and reviewed and/or edited the manuscript before submission

**Competing interests**

The San Raffaele Telethon Institute for Gene Therapy (SR-TIGET) is a joint venture between Fondazione Telethon and Ospedale San Raffaele (OSR). Gene therapies for ADA-SCID, Wiskott-Aldrich syndrome (WAS), metachromatic leukodystrophy (MLD), β-thalassemia (BTHAL) and Mucopolysaccharidosis I (MPSI) developed at SR-TIGET were licensed to Orchard Therapeutics (OTL) in 2018 and 2019. A. A. is the PI of the above clinical trials. A.J.T has equity in and is in the scientific advisory board for OTL and receives consultancy payments from Rocket pharmaceuticals.

**Peer review information**

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**Table 1. Ongoing clinical trials for monogenic disorders using ex vivo HSPC gene therapy.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Clinical trial registry numbers (trial phase)** | **Disease (defective gene)** | **Conditioning intensity and chemotherapy** | **Corrective strategy** |
| NCT03311503 (I/II), NCT03601286 (I/II) | SCID-X1 (*IL2RG)* | Low-dose busulfan | Autologous CD34+ cells transduced with the G2SCID lentiviral vector |
| NCT03217617 (I/II) | SCID-X1 (*IL2RG)* | Not known | Autologous CD34+ cells transduced with the self-inactivating lentiviral vector TYF-IL-2Rg |
| NCT01306019 (I/II) | SCID-X1 (*IL2RG)* | Low-dose busulfan | Autologous CD34+ cells transduced with the VSV-G pseudotyped lentiviral CL20- 4i-EF1a-hyc-OPT vector |
| NCT02999984 (I/II), NCT03765632 (I/II), NCT04140539 (II/III) | ADA-SCID (*ADA)* | Low-dose busulfan | Cryopreserved autologous CD34+ cells treated with an EFS-ADA lentiviral vector (OTL-101) |
| NCT03645460 (N/a) | ADA-SCID (*ADA)* | Not known | Autologous CD34+ cells transduced with the improved self-inactivating lentiviral vector TYF-ADA |
| NCT01515462 (I/II)  NCT03837483 (II) | WAS (*WAS)* | Reduced-intensity (busulfan and fludarabine) | Autologous CD34+ hematopoietic stem cells transduced ex vivo with a lentiviral vector carrying *WAS* (OTL-103) |
| NCT01410825 (I/II), NCT02333760 (I/II) | WAS (*WAS)* | Myeloablative (busulfan and fludarabine) | Autologous CD34+ hematopoietic stem cells transduced with the lentiviral vector w1.6W |
| NCT01347346 (I/II),  NCT01347242 (I/II) | WAS (*WAS)* | Myeloablative (busulfan and fludarabine) | Autologous CD34+ cells transduced with a lentiviral vector carrying *WAS* |
| NCT01855685 (I/II), NCT02234934 (I/II) | X-CGD (*CYBB)* | Myeloablative (busulfan) | Autologous CD34+ cells transduced with a lentiviral vector carrying human *gp91phox* (OTL-102) |
| NCT02757911 (I/II) | X-CGD (*CYBB)* | Myeloablative (busulfan) | Autologous CD34+ cells transduced with the G1XCGD lentiviral vector |
| NCT03812263 (I/II), NCT03825783 (I) | LAD (*CD18)* | Myeloablative (busulfan) | Autologous CD34+ cells transduced with the lentiviral vector Chim-CD18-WPRE, which carries the *ITGB2* gene |
| NCT03538899 (I/II) | ART-SCID (*DCLRE1C)* | Sub-myeloablative (busulfan) | Autologous CD34+ cells transduced with the self-inactivating lentiviral vector AProArt, carrying the corrected *DCLRE1C* gene |
| NCT02906202 (III), NCT03207009 (III) | TBT (*HBB)* | Myeloablative (busulfan) | Autologous CD34+ cells transduced with a lentiviral βA-T87Q-globin vector |
| NCT02453477 (I/II) | TBT (*HBB)* | Myeloablative (treosulfan and thiotepa) | Autologous CD34+ cells transduced with the GLOBE lentiviral vector (OTL-300) |
| NCT03655678 (I/II) | TBT (*HBB)* | Myeloablative (busulfan) | Gene editing of the erythroid enhancer of *BCL11A* using CRISPR-Cas9 to increase HbF expression |
| NCT03432364 (I/II) | TBT (*HBB)* | Myeloablative (busulfan) | Gene editing of the erythroid enhancer of *BCL11A* using zinc finger nucleases |
| NCT02151526 (I/II), NCT02140554 (I/II) | SCD (*HBB)* | Myeloablative (busulfan) | Autologous CD34+ cells transduced with a lentiviral βA-T87Q globin vector (LentiGlobin BB305 Drug Product) |
| NCT02186418 (I/II) | SCD (*HBB)* | Reduced intensity (melphalan) | Autologous CD34+ cells transduced with a γ-globin lentiviral vector (ARU-1801) |
| NCT03964792 (I/II) | SCD (*HBB)* | Not known | Autologous CD34+ cell transduced with the GLOBE1 lentiviral vector, which carries the βAS3 globin gene (DREPAGLOBE) |
| NCT02247843 (I/II) | SCD (*HBB)* | Myeloablative (busulfan) | Autologous CD34+ cells transduced with the Lenti/G-βAS3-FB lentiviral vector |
| NCT03282656 (I) | SCD (*HBB)* | Myeloablative (busulfan) | CD34+ cells transduced with a lentiviral vector containing a short-hairpin RNA targeting *BCL11A* to increase HbF expression |
| NCT04091737 (I) | SCD (*HBB)* | Reduced intensity (melphalan) | Autologous CD34+ cells transduced with a lentiviral vector encoding human γ-globin G16D and a short-hairpin RNA (RNA734) targeting the hypoxanthine guanine phosphoribosyltransferase gene *HPRT* (CSL200) |
| NCT03745287 (I/II) | SCD (*HBB)* | Myeloablative (busulfan) | Gene editing of the erythroid enhancer of *BCL11A* using CRISPR-Cas9 to increase HbF expression |
| NCT03157804 (I/II), NCT04069533 (II), NCT01331018 (I), NCT03814408 (I) | Fanconi anaemia type A (*FANCA)* | No conditioning | Autologous CD34+ cells transduced with a lentiviral vector carrying *FANCA* |
| NCT03351868 (N/a) | Fanconi anaemia type A (*FANCA)* | Not known | Autologous hematopoietic stem cells and mesenchymal stem cells transduced with a lentiviral vector carrying *FANCA* |
| NCT04105166 (I) | PKD (*PKLR)* | Not known | Autologous CD34+ cells transduced with a lentiviral vector containing *PKLR* (RP-L301) |
| NCT01560182 (I/II), NCT03392987 (II) | MLD *(ARSA)* | Myeloablative (busulfan) | Autologous CD34+ cells transduced with a lentiviral vector encoding human *ARSA* cDNA (OTL-200, Libmeldy) |
| NCT01896102 (II/III) | X-ALD *(ABCD1)* | Myeloablative (busulfan and cyclophosphamide) | Autologous CD34+ cells transduced with the self-inactivating lentiviral vector MNDprom-ABCD1 Lenti-D |
| NCT03852498 (III) | X-ALD *(ABCD1)* | Myeloablative (busulfan and fludarabine) | Autologous CD34+ cells transduced with the self-inactivating lentiviral vector MNDprom-ABCD1 Lenti-D |
| NCT03488394 (I/II) | MPSI *(IDUA)* | Myeloablative (busulfan and fludarabine) | Autologous CD34+ cells transduced with a lentiviral vector carrying human *IDUA* (OTL-203) |
| NCT04201405 (I/II) | MPSIIIA *(SGSH)* | Myeloablative (busulfan) | Autologous CD34+ cells transduced with a lentiviral vector carrying human *SGSH* |
| NCT03897361 (I/II) | Cystinosis *(CTNS)* | Myeloablative (busulfan) | Autologous CD34+ cells transduced with a pCCL-CTNS lentiviral vector carrying the human *CTNS* cDNA sequence |
| NCT02800070 (I), NCT03454893 (I/II) | Fabry disease (*GLA)* | Myeloablative | Autologous CD34+ cells transduced with the lentivirus vector AVR-RD-01 carrying *GLA* |

Data taken from a search performed on April 2020 for studies based on “hematopoietic stem cell” and “gene therapy” or “gene editing”, and “genetic diseases” on www.clinicaltrials.gov that are currently active (recruiting or not). Studies for which information has not been updated in the past 2 years have not been considered. SCID-X1, X-linked severe combined immunodeficiency; ADA-SCID, adenosine deaminase deficient severe combined immunodeficiency; N/a, trials without FDA-defined phases; WAS, Wiskott-Aldrich syndrome; X-CGD, X-linked chronic granulomatous disease; LAD, leukocyte adhesion deficiency; ART-SCID, ARTEMIS-deficiency severe combined immunodeficiency; TBT, transfusion-dependent β-thalassaemia; HbF, foetal haemoglobin; SCD, sickle cell disease; PKD, pyruvate kinase deficiency; MLD, metachromatic leukodystrophy; X-ALD, X-linked adrenoleukodystrophy; MPSI, mucopolysaccharidosis type I; MPSIIIA, mucopolysaccharidosis type IIIA.

**Figure 1. The haematopoietic hierarchy and genetic disorders.** Bone marrow resident hematopoietic stem cells (HSCs) and progenitor cells replenish blood and tissues with new mature cells. Both HSCs and haematopoeitic progenitor cells express the cell surface marker CD34, which is used to enrich for a mixture of hematopoietic stem and progenitor cells (HSPCs) for transplantation and gene therapy. HSCs can be classed as long-term hematopoietic stem cells (LT-HSC) or short term hematopoietic stem cells (ST-HSC). ST-HSCs progressively acquire lineage specifications in order to differentiate into lineage committed progenitors and eventually terminally differentiated cells, which are released into the peripheral blood. A simplified scheme of human haematopoiesis is presented here. Alternative models have been postulated based on cell surface marker analyses, in vitro and in vivo functional assays, clonal tracking by insertion analyses in HSPC gene therapy studies and single cell RNA analyses (reviewed in ref.22). Mendelian genetic disorders can affect self-renewal, differentiation and/or the function of different blood and immune cells. Examples of genetic diseases for which gene therapy is under investigation or are approved are represented in white boxes. MPP, multipotent progenitor; MEP, megakaryocytic-erythroid progenitor; CMP, common myeloid progenitor; GMP, granulomonocytic progenitor; LMMP, lymphoid-myeloid primed progenitor; CDP, common dendritic progenitor; CLP, common lymphoid progenitor; preB, pre-B cell; PreT, pre-T cell; NK, natural killer; SCID; severe combined immunodeficiency; CID, combined immunodeficiency. \*Wiskott-Aldrich syndrome affects platelets and other lineages.

**Figure 2. Timeline of progress of HSPC gene therapy**

Progress of HSPC gene therapy for monogenic disorders and enabling development in technology.

Data for figure:

|  |  |  |
| --- | --- | --- |
| **Year** | **Text** | **References** |
| 1980 | Development of recombinant viral vectors | 30,31 |
| 1990 | First gene therapy approved in humans, for the treatment of ADA-SCID using engineered peripheral blood lymphocytes | 9, 10, 12 |
| 1992-1995 | First gene therapy attempt using hematopoietic progenitors, employing bone marrow/cord blood progenitors for ADA-SCID | 9, 11 |
| 1996 | A self-inactivating lentiviral vector proves highly effective in transferring genes to HSPCs in preclinical studies | 32 |
| 2000-2004 | First demonstration of clinical benefit using HSPC gene therapy for SCID-X1 | 13, 89 |
| 2002 | First use of patient conditioning in HSPC gene therapy, using low-intensity conditioning in patients with ADA-SCID | 14 |
| 2003-2006 | Insertional mutagenesis observed in clinical trials for SCID-X1 linked to leukaemia cases | 16, 15 |
| 2006-2014 | Insertional mutagenesis after HSPC gene therapy in X-linked chronic granulomatous disease and Wiskott-Aldrich syndrome using gammaretroviral vectors limiting its clinical benefit | 110, 111 |
| 2009 | First clinical use of lentiviral vectors, for treatment of X-linked adrenoleukodystrophy | 33 |
| 2014 | First trials of gene editing in human T cells for the treatment of HIV-1 infection | 179 |
| 2013 | Demonstration of efficacy in HSPC gene therapy using lentiviral vectors for metachromatic leukodystrophy | 70 |
| 2013-2015 | Demonstration of efficacy in HSPC gene therapy using lentiviral vectors for Wiskott-Aldrich syndrome | 116, 91 |
| 2016 | HSPC gene therapy for ADA-SCID approved in the EU | 135 |
| 2018-2019 | Efficacy of HSPC gene therapy shown for β-thalassemia | 24, 25 |
| 2019 | HSPC gene therapy for β-thalassemia is approved in the EU | 181 |
| 2019 | First proof of principle study for the use of HSPC gene therapy in treating Fanconi Anemia | 27 |
| 2019 | First gene editing clinical trials start in human HSPCs | 182 |

**Figure 3. Manufacturing of engineered HSPC by gene addition and gene editing.** Autologous HSPCs are collected from the patient through multiple aspirations from the iliac crests or by leukapheresis following the administration of growth factor and chemokine antagonist. The collected material is enriched for CD34+ cells cultured in the presence of cytokines and genetically modified either by gene addition using gammaretroviral or lentiviral vectors or gene editing using zinc finger, CRISPR/Cas9 or transcription activator-like effector nuclease (TALEN) programmable endonucleases. Before gene therapy, patients are usually administered a conditioning preparatory regimen to deplete endogenous HSPCs. The intensity of conditioning ranges from reduced intensity to myeloablative, depending on the disease and the engraftment level required to reach the therapeutic threshold. The medicinal product is represented by the gene-corrected cells, ready for infusion at the end of the manipulation or after a cryopreservation and thawing step. Quality control tests performed on the drug product may include those on viability, sterility, endotoxin level, mycoplasma, immune phenotype, number of vector copies per genome, transduction efficiency, transgene expression vector, production contaminants and replication competent vector. In the case of fresh product or rapidly progressive diseases, a two-step strategy is employed to allow urgent treatment without completing all tests. Adapted from ref. X, Springer Nature Limited.

**Figure 4. HSPC gene therapy vector design and integration preferences**

**A)** Structure of the murine leukaemiavirus (MLV) gammaretrovirus and human immunodeficiency virus (HIV) lentivirus genomes and derived vectors. Gammaretroviral vectors (gRV) harbour a viral promoter in the U3 region of the 5’ long terminal repeat (LTR). In self-inactiving gammaretroviral (SIN-RV) and self-inactivating lentiviral (SIN-LV) vectors, deletions in the U3 region abolish the viral promoter and the expression of the therapeutic transgene is instead driven by a promoter — generally of cellular origin — placed between the two LTRs. Diseases for which each vector is being investigated and the corresponding therapeutic genes are shown. RRE; Rev responsive element. ***B)*** Differential distribution of MLV and HIV integration sites in the human genome is dictated by factors assembled in the pre-integration complex tethering the vector DNA to specific chromatin regions. MLV-derived vectors preferentially integrate into promoters and enhancers, whereas lentiviral vectors integrate into active gene bodies. Key proteins interacting with the MLV pre-integration complex (PIC) and influencing integration pattern of gRV and SIN-RV are the bromodomain/extraterminal domain proteins (BETs). The lens epithelium-derived growth factor (LEDGF/p75) mediates tethering of the lentiviral PIC to transcribed gene regions. Arrows indicate vector integration sites. Part B adapted with permission from ref. X, Elsevier.

**Figure 5. Gene editing techniques for HSPC gene therapy**

Molecular tools for gene editing include zinc finger nucleases, transcription activator-like effector nucleases (TALENs) and CRISPR-Cas9, which introduce targeted double-stranded DNA breaks (DSBs) into the genome. The cell repairs the DNA breaks by non-homologous end joining (NHEJ), potentially introducing small insertions and deletions (INDELs), or by homology-directed repair (HDR) if a suitable donor template is provided. Examples of gene editing approaches for the treatment of haemoglobinopathies are shown. HDR-driven strategies include correction of SNPs in the HBB gene locus to reverse red blood cell (RBC) sickling in sickle cell disease (SCD) or the targeted addition of HBB to treat either SCD or β-thalassaemia. NHEJ-driving strategies include the generation of mutations in the β-globin locus that mimic hereditary persistence of foetal haemoglobin (HPFH) and the inhibition of the expression of BCL11A, the main repressor of foetal HBG1/HBG2 genes, both resulting in the expression of foetal haemoglobin and amelioration of the disease phenotype. Elements of this figure are adapted with permission from ref X, Cell Press.

**Figure 6. HSPC‐driven localized delivery of therapeutics in lysosomal storage diseases.** Gene-corrected hematopoietic cells can release functional enzyme into the circulation and at a local level following migration into the tissue, such as the liver, spleen, bone, heart, kidney and peripheral nervous system (REF. 77, 164, 169, 170). In lysosomal storage enzyme diseases, substrate accumulation in cells of the central and peripheral nervous system leads to demyelination and cognitive and motor degeneration. In order to reach the CNS, cells need to cross the blood–brain barrier, which can be facilitated by chemotherapy (lightning bolt) and in some cases the underlying disorder. HSPCs may directly engraft in the CNS, expand locally and differentiate into corrected myeloid and microglia-like cells, which then release the therapeutic enzyme (REF. 78). In addition, corrected monocytes released from the bone marrow may migrate into the CNS, differentiate into macrophages and produce enzymes locally. The relative contribution of HSPC and mature cells to correction is still unclear (ref. 164). Therapeutic enzymes can be taken up by non-corrected cells expressing the mannose–phosphate receptor, including resident cells of the CNS, and break down accumulated substrates. Gene therapy may allow superior clearance of the accumulated substrates in the target organ compared with allogeneic HSPC transplantation, and replacement therapies that use enzymes that do not cross the blood–brain barrier.

**GLOSSARY**

Allogeneic

Relating to or denoting that the source of cells, tissues or organs for transplant is from a genetically different individual than the recipient.

Primary immunodeficiencies

Mendelian genetic disorders caused by defects in the development and/or function of immune cells. Currently, more than 300 genes have been identified that cause adaptive and/or innate immune cell defects.

Chronic granulomatous disease

Or CGD. A disease caused by dysfunction of the phagocyte NADPH-oxidase, a membrane-bound enzyme complex required for effective killing of bacteria and fungi.

Leukapheresis

A procedure that separates white blood cells including haemotopoeitic stem cells from the blood. White cells are collected from the donor and other blood fractions are returned to the circulation.

Mobilizing agents

Drugs that induce transient mobilization of haematopoeitic stem cells from the bone marrow to the circulation so that they can be collected by leukapheresis.

Myeloablative conditioning

High-dose chemotherapy that destroys hematopoietic cells in the bone marrow and severely reduces the number of blood cells. Usually followed by HSPC transplantation or gene therapy to rebuild the bone marrow.

Oligoclonality

A quality associated with clones derived from one or a few cells or molecules.

Iron chelation therapy

Pharmacological depletion of toxic iron accumulation in organs

Stress erythropoiesis

The rapid development of new red blood cells stimulated in response to acute anaemia