Gene therapy targeting haematopoietic stem cells for inherited diseases: progress and challenges

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Abstract | Pioneering gene therapy trials have shown that the genetic engineering of haematopoietic stem and progenitor cells can be an alternative to allogeneic transplantation in the treatment of primary immunodeficiencies. Early trials also highlighted the risk of insertional mutagenesis and oncogene transactivation associated with the first generation of gammaretroviral vectors. These events prompted the development of safer, self-inactivating lentiviral or gammaretroviral vectors. These lentiviral vectors have been successfully used to treat over 200 patients with 10 different haematological disorders (including primary immunodeficiencies, haemoglobinopathies and metabolic disorders) and for the generation of chimeric antigen receptor-T cells for cancer therapy. However, several challenges, such as effective reconstitution during inflammation, remain if gene therapy is to be extended to more complex diseases in which haematopoietic stem and progenitor cells can be altered by the disease environment. We discuss the progress made and future challenges for gene therapy and contrast gene therapy with gene-editing strategies.

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The use of retroviral vectors (particularly HIV-derived lentiviruses) for the ex vivo gene correction of haematopoietic stem and progenitor cells (HSPCs) constituted a breakthrough in the field of personalized medicine. Lentiviral vectors have enabled the rapid expansion of this approach even to complex genetic diseases (such as β-haemoglobinopathies) because these vectors can accommodate complex transcriptional units and transduce HSPCs with high efficiency. Furthermore, the integration profiles of lentiviral vectors are safer than those of gammaretroviral vectors[1]. No serious adverse events (SAEs) due to insertional mutagenesis have been reported more than 12 years after the start of the first lentiviral clinical trial — despite the administration of large numbers of transduced HSPCs (typically 5–20 million per kg of body weight) to more than 200 patients.

The clinical benefits reported to date are impressive, although the efficacy varies between diseases. These differences are related to disease-specific and human-specific pathophysiological obstacles that cannot be easily assessed in animal models. This inability to mimic the human disease in animal models means that phase I/II trials must be designed to optimize therapeutic strategies. Similar clinical results for the same disease have been obtained at different centres, confirming that this therapeutic strategy is more reproducible than is allogeneic HSPC transplantation (HSCT), for which intercentre differences in outcomes can be substantial. Improved reproducibility, which is associated with several other important parameters (such as patient acceptability and price), constitutes the basis for a profound change in the economic aspects of gene correction and is driving its wider application.

The results of several gene therapy trials in patients with primary immunodeficiencies[2–5], haemoglobinopathies[6,7] and inborn errors of metabolism[8,9] have recently been published. These results provide us with an opportunity to build a balanced picture of 30 years of effort in a field that lies at the interface between fundamental and clinical research. Gene therapy has been investigated for four primary immunodeficiencies; in two of those — X-linked severe combined immunodeficiency (SCID-X1) and SCID caused by adenosine deaminase ADA deficiency (ADA-SCID) — and for two non-immune diseases, metachromatic leukodystrophy (MLD) and certain subtypes of β-thalassaemia, gene therapy can now successfully replace stem cell transplantation from allogeneic donors. The choice of a gene therapy option for these patients must always be carefully balanced against the improvements in disease-free survival and quality of life obtained with more conventional procedures, such as allogeneic HSCT.

Hence, the objectives of our Review are to assess this balance in light of the most recently published results and to identify bottlenecks that restrict the use of human gene therapy.

Viral vectors for HSPC engineering

A number of retroviral genera have been adapted for use as ex vivo gene transfer vectors; they include the gammaretroviral vector derived from Moloney murine leukaemia virus[10], as well as lentiviral vectors derived from HIV-1 (REFS[11,12]), which are currently the most popular. These retroviral vectors allow delivery of up to 8 kb of transgene to HSPCs, followed by stable genomic integration of the vector, which enables permanent expression of the transgene in the blood progeny cells. This is in contrast with adeno-associated viral vectors, which are nonintegrating vectors and are predominantly used for in vivo gene therapy that targets nondividing postmitotic cells — adeno-associated viral vectors have successfully been used in the treatment of haemophilia and eye diseases[13]. Here, we focus on clinical trials based on retroviral vectors for ex vivo HSPC engineering.

Gene therapy: immunodeficiencies

It has been 20 years since the first gene therapy trials, for SCID, proved the curative potential of ex vivo gene addition to HSPCs[14]. Severe combined immunodeficiency is caused by profound defects in immune system development and function. As a consequence, children with SCID are susceptible to severe, life-threatening infections. The condition is genetically heterogeneous, and approximately 20 different genetic causes have been identified to date[15]. Two of these (SCID-X1 and ADA-SCID) account for 40% and 10% of all SCID forms, respectively. Given that all forms of SCID are characterized by the absence of circulating, functional, polyclonal T cells, untreated patients typically die from opportunistic infections during their first year of life. Both SCID-X1 and ADA-SCID have been successfully treated with gene therapy. A gene therapy product for ADA-SCID is the second gene-based product to have obtained marketing approval from the European Medicines Agency (Strimvelis; GlaxoSmithKline).

Severe combined immunodeficiency caused by adenosine deaminase deficiency. The principal characteristic of ADA-SCID is the systemic nature of this purine metabolism disorder; the patient experiences a wide range of non-immune complications in the pulmonary, haematological, gastrointestinal, neurological and skeletal systems[16]. The treatment options for ADA-SCID are enzyme replacement therapy (ERT), HSCT from a sibling donor with a genetically identical human leukocyte antigen (HLA) and, if available, gene therapy. At present, retrovirus-based gene therapy is associated with an overall survival of 100% and an efficacy rate (defined as the cessation of ERT and the avoidance of allogeneic transplantation) of approximately 80%[17]. The efficacy rate is higher in the most recent clinical trial[18], which used a lentiviral vector. Over the past 20 years, more than 100 patients with ADA-SCID have been treated in various gene therapy trials. The four main lessons from these trials are outlined in the rest of this section.

First, low-dose busulfan for patient conditioning before the infusion of gene-modified HSPCs is required for subsequent engraftment.

Second, the use of ERT before cell infusion and 1 month after infusion does not blunt the putative selective advantage of ADA-replete cells. On the contrary, ERT may even improve the outcome of gene therapy through three mechanisms: partially correcting the hypocellularity within the bone marrow (which leads to more efficient haematopoietic stem cell (HSC) mobilization in the blood), shortening the period of lymphopenia (before the development of new lymphocytes from the gene-corrected graft) and protecting against systemic organ toxicity (notably damage to the thymic and pulmonary epithelia)[19]. Given that the bone marrow is damaged by the accumulation of toxic metabolites, the performance of a myelogram before HSC harvesting is recommended. This may enable the detection of pre-existing cytogenetic abnormalities, which would constitute a potential limitation for patients scheduled for autologous gene transfer[20]. This limitation also applies to other complex inherited diseases (see below).

Third, there is still room for improving quality of life in patients with ADA-SCID who are treated with gene therapy or allogeneic HSCT. The presence of persistent neurological, auditory and behavioural problems in patients after both procedures suggests that, even in the presence of systemic detoxification, blood-derived ADA-expressing cells that cross the blood–brain barrier do not deliver sufficient levels of ADA for the full correction of metabolic alterations in the brain[21]. Alternatively (and not mutually exclusively), we can also hypothesize that restoration of ADA expression is inefficient because of permanent central nervous system (CNS) damage. Direct delivery of ADA to the CNS using in vivo gene therapy might be worth exploring, as was recently proposed for other metabolic disorders[22,23].

Fourth, the ADA-SCID trials are the only gammaretrovirus-based gene therapy trials in which SAEs due to insertional mutagenesis have not been observed (TABLE 1). However, owing to the cellular proliferation driven by insertional mutagenesis by first-generation gammaretroviral vectors in three other clinical trials in patients with immunodeficiency, caution is warranted. A self-inactivating (SIN) retrovirus-based vector (lentiviral or gammaretroviral), in which expression of the therapeutic gene is controlled by an internal promoter (FIG. 1), should be used preferentially over a gammaretroviral vector with a functional long terminal repeat (LTR). The term 'self-inactivating' comes from the design of the vector system: a deletion is introduced into one of the LTR sequences of the vector. This deletion is then present on (and inactivates) both LTRs after one round of transcription and reverse transcription. The use of a SIN lentiviral vector in new trials in the UK and USA was associated with successful reconstitution in all 61 treated patients[18] (H. B. Gaspar, personal communication); these results compare favourably even with HLA-genoidentical HSCT[18].

Haematopoietic stem and progenitor cells
(HSPCs). A heterogeneous cell population that can be isolated using the surface marker CD34 and comprises both the most immature haematopoietic stem cells responsible for long-term engraftment and haematopoietic progenitors that have lost self-renewal capacity, are more restricted in term of lineage potential and are responsible for short-term engraftment.

Enzyme replacement therapy
(ERT). A medical treatment aiming to replace a missing protein. In the case of severe combined immunodeficiency caused by adenosine deaminase (ADA) deficiency, pegademase bovine ADA is used.

Patient conditioning
The treatments used to prepare a patient for haematopoietic stem and progenitor cell transplantation. The conditioning regimen may include chemotherapy, monoclonal antibody therapy and radiation. It helps make room in the patient's bone marrow for new haematopoietic stem cells and to prevent rejection in case of allogeneic transplantation.

Haematopoietic stem cell
(HSC). A cell defined by the capacity to self-renew and the ability to ensure continuous production of all blood lineages for the entire life of an individual.

Myelogram
This bone marrow puncture is a medical test that consists of taking a bone marrow sample from the hip or the sternum. Once the extract has been smeared onto slides, the laboratory analyses the cellular composition of the sample.

Table 1 | **HSPC-based gene therapy trials using LTR-driven gammaretroviral vectors**

Disease	Trial number (phase)	Starting year; site	Vector	Conditioning	Number of patients	SAEs (deaths)[a]	Refs
SCID-X1	NA	1999; France	*g*RV-IL-2Rγ	No	10	5 (1)	14,29
SCID-X1	NA	2002; UK	*g*RV-IL-2Rγ	No	10	1	26,30
WAS	DRKS00000330 (phase I/II)	2006; Germany	*g*RV-WAS	Yes	10	9 (3)	45
ADA-SCID	NCT00599781 (phase I/II)	2000; Italy	*g*RV-ADA	Yes	22	0	17,18,164
ADA-SCID	NCT03478670 (phase IV)	2017: Italy	*g*RV-ADA (Strimvelis)	Yes	5	0	b
ADA-SCID	NCT00018018 (phase I/II)	2001: USA	*g*RV-ADA	Yes (6); no (4)	10	0	165
ADA-SCID	NCT00794508 (phase II)	2009; USA	*g*RV-ADA	Yes	10	0	166
ADA-SCID	NCT01279720	2003; UK	*g*RV-ADA	Yes	8	0	28
CGD	NCT00564759	2004; Germany	*g*RV-CYBB	Yes	2	2 (1)	167,168
CGD	NCT00927134	2004; Switzerland	*g*RV-CYBB	Yes	1	1	169

ADA, adenosine deaminase; ADA-SCID, ADA severe combined immunodeficiency; CGD, chronic granulomatous disease; gRV, gammaretrovirus; HSPC, haematopoietic stem and progenitor cell; IL-2Rγ, interleukin-2 receptor subunit-γ; LTR, long terminal repeat; NA, not available; SAEs, serious adverse events; SCID-X1, X-linked SCID; WAS, Wiskott–Aldrich syndrome. [a]Related to the drug product. [b]Personal communication from A. Aiuti.

X-linked severe combined immunodeficiency. Naturally occurring mutations in the gene encoding interleukin-2 receptor subunit-γ (*IL2RG*) are responsible for SCID-X1. This condition is characterized by the complete absence of T cells and natural killer (NK) cells, whereas B cells are present but functionally impaired. Before the advent of gene therapy, the only curative treatment was HSCT. The latter results in a favourable outcome when an HLA-compatible donor is available (>90% chance of success) or when, in the absence of a genoidentical donor, SCID-X1 is diagnosed and treated with HSCT from a haploidentical donor before the development of active infections and the associated inflammation. This favourable outcome is due to the availability of empty thymic niches and the absence of NK cells, which are responsible for rejection in patients with forms of SCID that lack T and B cells but still have NK cells. The use of alternative donors in patients without infection can achieve >10-year survival outcomes similar to those of patients with matched donors[24,25]. However, the proportion of those surviving >10 years is only 50% in patients with active viral or mycobacterial infections because of delayed (>6 months) restoration of full cellular immunity and/or the occurrence of graft-versus-host disease. Moreover, full immune reconstitution is rarely observed with alternative donors — probably because of alloreactive damage to the thymus, which is present even in the absence of any clinical signs of graft-versus-host disease.

In the first two proof-of-principle gene therapy trials, conducted in France and the UK, 20 infants with SCID-X1 and no matched sibling donor were treated (in the absence of any conditioning regimen) with an infusion of autologous $CD34^+$ bone marrow cells after transduction with a gammaretroviral LTR-driven vector[14,26]. The two trials demonstrated not only the enormous therapeutic potential of this approach but also the great risk associated with first-generation retroviral vectors[27]. Almost 20 years after gene transfer, 18 of the 20 treated patients are alive and have full or nearly full correction of T cell immunodeficiency, including normal T cell subset counts, the sustained presence of naive T cells (even in patients who were treated for leukaemia after gene therapy), a diversified T cell repertoire and normal T cell-mediated immune functions[2,28] (M.C., unpublished data; H. B. Gaspar and A. J. Thrasher, personal communication). Nevertheless, the occurrence of T cell leukaemia in six patients (between 2.5 years and 15 years post-therapy, of whom one died)[29,30] prompted the discontinuation of these trials and the development of safer vectors (discussed below).

More recently, the third international SCID-X1 gene therapy clinical trial (performed in parallel in Europe and the USA) was conducted with a second-generation SIN retroviral vector devoid of any LTR enhancer sequences[31]. Seven of the nine treated patients recovered a functional T cell compartment with sustained immune function and no genotoxic effects. Integration site analysis revealed a significant reduction in the number of integration sites clustered close to proto-oncogenes such as *LMO2* or *MECOM* (also known as *MDS1* and *EVI1* complex locus) — demonstrating improved safety. However, the overall retroviral integration pattern remained similar, with a preference for active promoters and enhancers. This contrasts with the findings for lentiviral vectors, which have potentially safer integration patterns[1]. Furthermore, the great variability in the degree of retroviral transduction observed in the SIN retroviral trial and the increased number of stem cells corrected with use of a lentivirus justified the transition to SIN lentiviral vectors in two ongoing clinical trials.

The first such vector was developed by B. Sorrentino's group. The SIN lentiviral vector uses an elongation factor 1α (EF1α) promoter to drive a codon-optimized human γ-chain cDNA flanked by two LTRs containing a 400 bp hypersensitive site 4 (HS4) chromatin insulator sequence from the chicken β-globin locus (a 250 bp core HS4 insulator plus a 3′ flanking sequence)[32,33]. The insulator blocks the transcriptional activation of nearby genes by elements in the lentiviral vector. The vector is produced by the first stable trans-complementing cell line described in the literature[34]; this stable producer

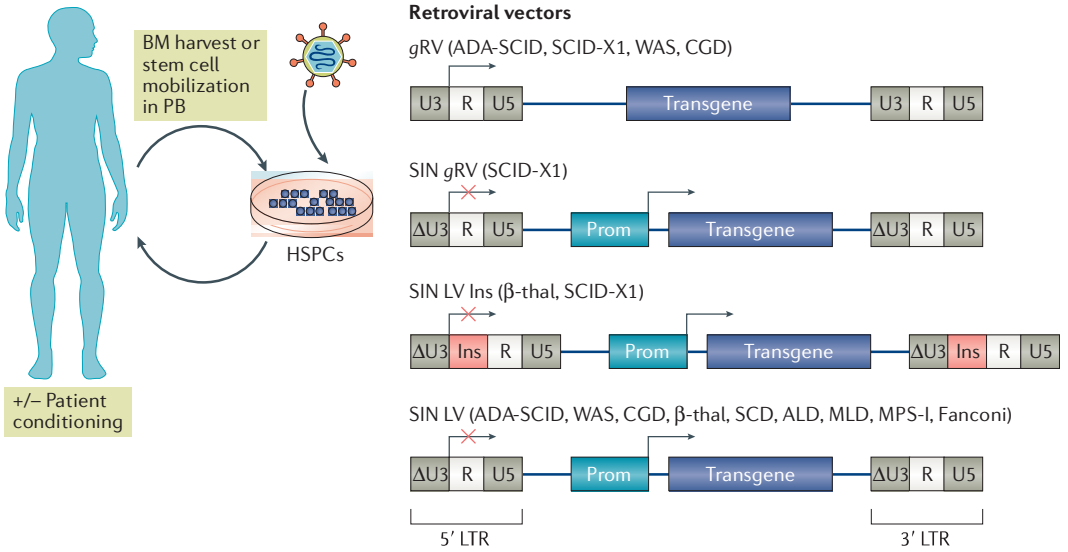

Fig. 1 | **Ex vivo gene therapy for inherited blood disorders.** Haematopoietic stem and progenitor cells (HSPCs) are harvested from bone marrow (BM) or mobilized into peripheral blood (PB) and collected by apheresis. After transduction with retroviral vectors, HSPCs are reinfused into the patients who have usually received a partial or full myeloablative conditioning regimen. Of note, patients did not undergo conditioning in the gammaretrovirus (*g*RV)-based gene therapy trials for X-linked severe combined immunodeficiency (SCID-X1). Past and ongoing clinical trials using the different types of retroviral vector are reported in brackets. First-generation gammaretroviral vectors harbour wild-type 5′ and 3′ long terminal repeats (LTRs) containing U3, R and U5 regions. The U3 region containing potent enhancer and/or promoter elements is deleted in self-inactivating (SIN) retroviral vectors (ΔU3). β-thal, β-thalassaemia; ADA-SCID, adenosine deaminase SCID; ALD, adrenoleukodystrophy; CGD, chronic granulomatous disease; Fanconi, Fanconi anaemia; Ins, hypersensitive site 4 chromatin insulator; LV, lentivirus; MLD, metachromatic leukodystrophy; MPS-I, mucopolysaccharidosis type I; Prom, promoter; SCD, sickle cell disease; WAS, Wiskott–Aldrich syndrome.

can provide up to 150 L of vector in a single production run — solving an important problem for the wide-scale treatment of adult patients.

In collaboration with Malech at the US National Institutes of Health (NIH), Sorrentino's group made a major step forward in SCID-X1 gene therapy. A significant clinical improvement was obtained in two older patients (aged 22 and 23 years) affected by persistent immune dysfunction despite haploidentical HSCT in infancy. Both patients were treated with low-dose busulfan before gene therapy[35]. These advances followed the earlier failure of retroviral gene therapy in two patients aged 3 and 15 years as part of the first such trials in France and the UK; this failure was probably due to the absence of conditioning and, for the 15-year-old, a non-functional thymus[36]. Two findings from this new group of patients were particularly important: an elevated number of T cell receptor excision circles (indicating the presence of at least some residual thymus function, despite the patients' age) was observed, as was the complete correction of the B and NK cell compartments, which is associated with the use of low-dose busulfan[35]. Malech's group (for patients aged >2 years) and Sorrentino's group (for newborns)[37] demonstrated that conditioning with very low doses of busulfan is associated with low toxicity and a high level of gene-corrected B cells. Further advantages of B cell correction include the absence of a requirement for immunoglobulin replacement therapy and the prevention of bronchiectasis and the chronic upper respiratory tract infections and inflammation that complicate long-term outcomes in these patients.

It is important to note that even for gene therapy (autologous transplantation, where no alloreactivity is present), the presence of generalized BCGitis or a severe viral infection results in significantly delayed, less robust T cell immune reconstitution[31]. The twofold risk associated with the infectious context (mortality and delayed, poor T cell reconstitution) justifies the search for methods capable of accelerating the thymic production of T cells and circumventing the limitation on thymic function. One of these methods is the infusion of ex vivo generated T precursor cells in order to bypass the first steps of intrathymic differentiation. This approach will be tested in a phase I/II clinical trial at Necker Children's Hospital, Paris, France, in 2019. These T precursor cells can be obtained from any source of $CD34^+$ HSPCs (mobilized peripheral blood or cord blood) in a 1-week in vitro stromal cell-free culture system that uses the Delta-like protein 4 (DLL4)–Notch signalling pathway and pro-T cell cytokines[38,39]. Furthermore, the T precursor cells can be genetically modified using a lentiviral vector (I.A.-S., unpublished results). The clinical trial will include patients with SCID undergoing haploidentical HSCT. In addition to the standard non-manipulated graft, each patient will receive a single dose of T cell precursors generated from donor HSPCs. If the preclinical results in murine models of SCIDs[39] are confirmed in the clinic, this procedure should solve the problem of the delayed immune reconstitution for both

Bronchiectasis
A form of chronic lung disease defined as the abnormal irreversible dilatation of the bronchi in which the elastic and muscular tissues are destroyed by acute or chronic inflammation and infection.

BCGitis
Regional lymphadenitis, a severe disseminated disease, following bacillus Calmette–Guérin vaccination.

partially HLA-compatible HSCT and the autologous transplantation of genetically modified cells.

The treatment algorithm in SCID-X1 is less clear than that described in ADA deficiency, where the toxic effects of the accumulated metabolites and the need for intracellular detoxification limit the long-term benefit of ERT and are responsible for high mortality and morbidity in recipients of non-genoidentical transplants. In SCID-X1, the early implementation of a screening programme may enable allogeneic transplantation to be performed before the occurrence of any infectious episodes. Given the recent progress in allogeneic HSCT (in the favourable SCID-X1 setting, which is characterized by a lack of T and NK cells and the absence of systemic organ toxicity), the risk–benefit ratio for gene therapy, compared with that of allogeneic HSCT, must be carefully evaluated. Our recent comparison of ten patients who underwent haploidentical HSCT with patients treated with gene therapy argued strongly in favour of gene therapy (if available); we observed substantially faster and more robust T cell reconstitution, which persisted up to 4 years after the infusion of the genetically modified cells[40]. Of course, these results must be confirmed in a larger set of patients and assessed with regard to the progress being made in both gene therapy and transplantation and the continuing absence of vector-related SAEs.

Other immunodeficiencies: challenges to be solved. These early, positive results prompted the use of gene addition therapy in two other primary immunodeficiencies: Wiskott–Aldrich syndrome (WAS) and X-linked chronic granulomatous disease (X-CGD). Promising but preliminary results have been obtained in both contexts, even though the pathophysiology is certainly more complex in these diseases than in SCID-X1 and ADA-SCID — explaining the need for further improvements.

Wiskott–Aldrich syndrome protein (WASP) is required for cytoskeletal reorganization, signal transduction and terminal differentiation in several haematopoietic cell types. Hence, mutations in the gene (*WAS*) cause a complex, X-linked primary immunodeficiency that has many clinical manifestations, including microthrombocytopenia, eczema and recurrent infections. Patients also have a tendency to develop autoimmune manifestations and tumours (for a review, see REFS[41,42]). A genotype–phenotype correlation has been reported[43].

After extensive in vivo and in vitro studies, the first clinical gene therapy trial (conducted in Hannover) enrolled ten patients and treated them with a WASP-encoding, LTR-driven first-generation gammaretroviral vector[44]. The reconstitution failed in one patient, and the other nine patients had complete reconstitution of their immune system but developed myelodysplastic syndrome or leukaemia at different time points[45] (C. Klein, personal communication); this raised serious concerns about this first-generation vector. Following further preclinical studies, a subsequent clinical trial based on a lentiviral vector encoding the human WASP cDNA under the control of the human endogenous promoter was performed in Milan[3], Paris, London[4] and (most recently) Boston[46].

The results obtained in the European centres were similar. In particular, T and B cell immunity was well restored because of the strong selective advantage of WASP expression in these cell lineages. As a consequence, 12 months after gene therapy, patients were free of recurrent infections and had substantially less frequent autoimmune episodes, which are two of the three life-threatening symptoms responsible for a poor prognosis in untreated patients (the third is profound microthrombocytopenia).

Nevertheless, there is room for improvement. Despite a myeloid engraftment rate as high as 50% (the threshold established in allogenic HSCT for correction of the profound microthrombocytopenia seen in WAS) in most patients, the platelet count remained abnormally low. Although bleeding episodes are stopped in all patients, the platelet count is still too low to prevent acute bleeding (for example, during or after surgery). More detailed studies are ongoing, which aim to determine the reasons for this partial correction of the platelet compartment. This life-threatening characteristic justifies giving the patients the most severe possible Ochs score of 5 (REF.[47]).

An important clue about how to improve therapy may be in the difference between the vector copy number (VCN) in the drug product (the transduced cells before transplantation) and the VCN detected in the circulating blood cells after transplantation[3,4]. A decrease in the VCN was observed in the WAS trial and has also been observed in trials for X-CGD and X-linked adrenoleukodystrophy (X-ALD). There are several possible, non-mutually exclusive explanations for the discrepancy between the VCN in the drug product and the VCN in engrafted cells. First, the high transduction rate might reflect transduction of precursor cells that are lost over time and not the transduction of true stem cells. Second, the most highly transduced HSCs might die in vivo — perhaps after the activation of mechanisms that sense infectious particles. Lastly, disease-specific characteristics (such as poor migration in WASP-deficient stem cells) might cause poor mobilization of true stem cells during HSPC collection and/or poor uptake of genetically corrected cells (in the event of suboptimal correction). This latter aspect might be solved by optimizing the transduction conditions (see the section below on the transduction procedure and the stem cell source).

This problem was particularly notable in several attempts to correct CGD by combining gene addition via retroviral vectors with bone marrow conditioning. CGD is a primary immunodeficiency of innate immunity caused by defects in phagocyte NADPH oxidase subunits. Loss-of-function mutations in the NADPH oxidase components (that is, X-linked GP91PHOX and autosomal recessive P22PHOX, P67PHOX (also known as NCF2) or P47PHOX (also known as NCF1)) abrogate oxidase activity and compromise host immunity against certain bacteria and fungi. All the X-CGD gene therapy trials reported low levels of long-term engraftment and transient clinical benefit despite a high VCN in the drug product and high numbers of reinfused cells (for a detailed review, see REF.[48]). More recently, new clinical

Ochs score
For Wiskott–Aldrich syndrome, the widely used clinical severity score developed by Ochs (ranging from 1 to 5). A score of 5 is associated with severe disease (autoimmunity, infections, inflammation and/or malignancy).

Vector copy number
(VCN). The average number of integrated therapeutic vector copies per cell in a given population. The VCN can be used to evaluate the transduction and/or correction level in this population.

trials for X-CGD have been initiated in Europe and in the USA and treated nine patients so far[49] (M.C., unpublished results); these trials use a chimeric myeloid-specific promoter to express *CYBB*, which encodes the NADPH oxidase catalytic subunit cytochrome *b*-245 heavy chain (also known as GP91PHOX)[50,51]. Since the publication of results from these trials, several studies have reported that chronic inflammation negatively affects HSPCs in CGD and in other contexts. In both mice and humans with X-CGD, there was a clear reduction in the proportion of HSCs in the bone marrow[52]. Furthermore, these HSCs showed rapid exhaustion after in vitro culture (HSCs from humans) and increased cycling and impaired long-term engraftment potential (HSCs from mice); in both cases, high levels of pro-inflammatory cytokines such as IL-1β were observed[52,53]. The responses of HSCs to chronic inflammation are similar to those described in settings linked to ageing or infections[54–58]. The responses are due — at least in part — to the direct sensing of pathogens and inflammatory molecules by pattern recognition receptors such as Toll-like receptors and cytokine and chemokine receptors[59–64]. Inflammation — whether due to infection or other causes — modulates the functions and potential of stem cells[65]. This inflammatory context is particularly problematic for autologous gene therapy approaches and requires specific optimization of the transduction process (see below). Several research groups are working intensively to determine whether this problem can be solved by in vivo or ex vivo treatment with anti-inflammatory drugs. The resolution of this obstacle will determine whether gene therapy can successfully replace HSCT from allogeneic donors for patients with CGD.

Gene therapy: other monogenic diseases

Lysosomal storage diseases. Gene therapy for lysosomal storage diseases has yielded impressive clinical results, especially for MLD, which is caused by a defect in the production of a functional lysosomal enzyme, arylsulfatase A (ARSA). Although ERT and HSCT have been evaluated as potential treatments for lysosomal storage diseases, the preclinical and clinical results have shown limited efficacy in most cases. In particular, patients receiving transplants after symptom onset did not show clinical benefit after transplantation, and mortality was substantial.

The current gene therapy approach for MLD is based on the transplantation of autologous HSPCs that differentiate into macrophages and microglia in the CNS and then provide the ARSA for cross-correction of the affected nervous tissue. After promising results were obtained in an MLD mouse model[66,67], a phase I/II clinical trial of gene therapy for MLD was started in Milan in 2010. After a median follow-up period of 36 months, the results for the first nine patients showed haematopoietic reconstitution in all instances, stable engraftment of gene-corrected cells and stable reconstitution of ARSA activity in the cerebrospinal fluid as early as 6 months after gene therapy[8,9]. The recovery of ARSA activity in the cerebrospinal fluid indirectly shows that genetically corrected HSC-derived cells had migrated to the CNS and produced the enzyme locally. Moreover, the study's results emphasized that better outcomes were obtained in children treated before or very soon after symptom onset, leading to better maintenance of motor and cognitive functions and the prevention or slowing of CNS demyelination. With a view to further increasing treatment efficacy in this context, a combination of HSPC-based gene therapy and intracerebral gene delivery is expected to reduce the lag in enzyme delivery to the CNS after HSC-based gene therapy alone. Several procedures for intracerebral gene delivery have been developed. Recently, experiments in two different immunodeficient murine models showed that intracerebroventricular transplantation of human HSPCs, transduced with a therapeutic ARSA-expressing lentiviral vector, resulted in the more effective and stable delivery of higher levels of ARSA enzyme to the brain relative to standard intravenous transplantation[8]. Furthermore, the combination of intracerebral and intravenous transplantation led to even more consistent engraftment of human gene-modified HSPCs[68]. This approach might be extremely beneficial for a number of lysosomal storage diseases, might avoid the rapid disease progression observed during the 6 months after conventional transplantation (due to the required conditioning regimen) and might also improve the clinical results reported for other metabolic diseases such as X-ALD[69].

β-Haemoglobinopathies. The β-haemoglobinopathies β-thalassaemia and sickle cell disease (SCD) are the most common monogenic diseases worldwide and constitute a global health problem.

β-Thalassaemia is caused by mutations that reduce (β^+, including the β^E genetic variant) or abolish (β^0) the synthesis of β-globin chains[70]. The excess of noncoupled α-chains leads to ineffective erythropoiesis, intramedullary haemolysis and haemolytic anaemia. The clinical severity varies as a function of the disease-causing mutations and the concomitant presence of α-thalassaemia or compensatory mechanisms (such as the persistence of fetal β-like γ-globin)[70]. Patients with clinically severe β-thalassaemia present with anaemia, iron overload, hepatosplenomegaly and various organ complications, the severity of which depends on the adequacy of supportive treatment (typically monthly blood transfusions and iron chelation). At present, the only curative treatment for β-thalassaemia is allogeneic HLA-genoidentical HSCT. The quality of the outcome is closely related to the age at transplantation, the presence or absence of alloimmunization and the severity of organ damage. HSCT requires high-dose chemotherapy and immunosuppression.

For the past 20 years, gene therapy has been investigated as an alternative curative treatment for all patients with β-haemoglobinopathies — the vast majority of whom lack a compatible sibling donor for HSCT. After the seminal discovery of the genomic elements that control β-globin gene expression, a major breakthrough came with the generation of SIN lentiviral vectors that feature an optimized β-globin gene under the control of the β-globin promoter, a 3′ enhancer and the DNase-I-hypersensitive sites 2, 3 and 4 from the β-globin locus control region.

The first trial of gene therapy for β-thalassaemia (the LG001 study, authorized in France in 2006) employed a lentiviral vector (HPV569) containing a β-globin cassette flanked by two LTRs, each containing two copies of the core 250 bp HS4 chicken insulator[71]. The first patient to be treated became transfusion-independent 1 year after cell infusion[72]; the blood haemoglobin levels remained stable at around 8.5 g/L for more than 8 years, with the therapeutic haemoglobin accounting for 30% of the total haemoglobin. A dominant clone with an insertion inside the *HMGA2* gene was detected a few months after transplantation. The abundance of this clone reached a plateau between years 1 and 3, and declined thereafter. The clone is now ranked only fifth in terms of its contribution to the cell population in this patient[6].

This clinical trial provided proof of principle for the correction of β-thalassaemia by gene addition. It paved the way for subsequent improvements and phase I/II trials worldwide. Two crucial improvements, prompted by these initial results, were made in vector choice and patient management before HSPC harvesting. Because the chromatin insulator within the HPV569 lentivirus decreased the titre and transduction efficiency of the vector and was also responsible for genetic instability, Leboulch's group removed it and introduced the cytomegalovirus promoter to drive transcription of the new BB305 vector in packaging cells[73]. The first study (LG001) showed that HSC harvesting was a clear bottleneck; the patients' bone marrow was strongly biased towards cells committed to the erythroid lineage. A special treatment regimen improved the number of HSPCs harvested; the regimen consisted of a 3-month hypertransfusion regimen (with careful monitoring of the serum transferrin receptor level), a mobilization regimen combining granulocyte colony-stimulating factor (G-CSF) and plerixafor (with careful monitoring of the peripheral $CD34^+$ cell count) and full myeloablative busulfan-based conditioning (leading to engraftment in all patients in the HGB-205 trial)[6].

The results published for 22 patients in two phase II trials (HGB-205 and HGB-204) suggest that this established clinical protocol and vector are of therapeutic value in patients with β^E/β^0 thalassaemia, who residually express a functional β-globin chain (from the β^E genetic variant) and account for approximately 50% of cases of transfusion-dependent β-thalassaemia. Almost all the patients achieved sustained transfusion independence. In a few of these cases, the haemoglobin level achieved or approached normal values for healthy individuals and thereby also corrected dyserythropoiesis. Conversely, only three of nine patients with β^0/β^0 thalassaemia or patients who were homozygous for the IVS110 mutation (a β^0 genotype with only trace endogenous β-globin expression) achieved transfusion independence; nevertheless, the requirement for transfusion was reduced in the other six cases[6].

Another phase I/II clinical trial in transfusion-dependent β-thalassaemia started in Italy in 2015 (NCT02453477); it was based on transplantation of HSPCs, mobilized with G-CSF and plerixafor and transduced with the compact β-globin-expressing GLOBE vector[74,75]. A myeloablative, reduced-toxicity, conditioning regimen (based on treosulfan and thiotepa) was used to favour engraftment while ablating extramedullary haematopoiesis. As of December 2016, seven patients with different genotypes had been treated with plerixafor plus G-CSF-mobilized, transduced $CD34^+$ cells at a high dose ($>10 \times 10^6$ cells per kg) and a VCN per cell ranging from 0.7 to 1.5. The clinical outcome to date indicates a large reduction in the transfusion requirement, with greater clinical benefits in younger patients[76].

Gene therapy for β^0/β^0 thalassaemia requires further improvements and the evaluation of various parameters: patient management before harvesting and transplantation, vector production, HSPC transduction efficiency, the dose of genetically corrected HSCs per kg, the degree of conditioning and/or myeloablation needed and accurate analyses of the stem cell compartment and the exhaustion status of true stem cells. The injection of a drug product containing 10×10^6 highly purified $CD34^+$ cells (transduced at 1 VCN) per kg into a patient after full myeloablation should enable correction (or at least transfusion independence) in patients with β^0/β^0 thalassaemia without any need to increase the VCN per cell, which could be dangerous[6] (M.C., unpublished data).

In SCD, the substitution of valine for glutamate at position 6 of the β-globin protein is responsible for deoxygenation-induced haemoglobin S polymerization. This primary event drives red blood cell sickling, haemolysis, an increase in blood viscosity, vaso-occlusive crises, stroke and multi-organ damage (for a detailed review, see REF.[77]). A complete correction of the clinical phenotype was observed in a patient with SCD who received 5×10^6 $CD34^+$ cells per kg, with a VCN of 1 (the HBG205 clinical trial using the BB305 vector)[7]. This patient has a therapeutic β-globin level of around 50%, no longer receives blood transfusions and has had a stable clinical profile (similar to that of a heterozygous sickle cell haemoglobin (HbS) carrier) for 4 years. In a subsequent study (HBG206), however, Kanter et al.[78] obtained only a low level of gene transduction and found no clinical benefit in patients with SCD who received only 2×10^6 $CD34^+$ cells per kg, with a median VCN of 0.6. This finding again highlights the importance of monitoring the stem cell source and the transduction efficiency of the autologous genetically modified graft. Novel methods for stem cell collection and HSPC transduction were recently tested in the same HBG206 trial, with encouraging early results[79,80]. Other clinical trials addressing this global health burden are ongoing, although the results are not yet available (NCT02186418 and NCT02247843).

Current challenges in transduction

The occurrence of multiple SAEs in several clinical trials with gammaretroviral vectors (except for in ADA-SCID) focused efforts on developing safe vectors. This work has been successful; no SAEs caused by insertional mutagenesis by lentiviral vectors have been reported. Recent research has focused on improving the transduction process itself and on characterization of lentivirus-transduced cells.

To date, the conventional HSPC transduction procedure has been driven by the initial need (for transduction

with gammaretroviral vectors) for the HSPCs to be in the cell cycle[14]. This pioneering protocol has barely been modified, even though lentiviral vectors integrate into active HSPCs and do not require cycling cells for gene transfer.

Culture conditions. Indeed, a growing body of experimental evidence has shown that cultured HSPCs progressively lose their engraftment capacity as a result of recruitment into the cell cycle. The HSPCs shed adhesion molecules during growth and culture (which impedes their homing to the appropriate niche) and show greater lineage commitment and differentiation[81–83]. The ex vivo cell culture time is correlated with the level of transcriptional modifications and the engraftment capacity of the cells, both of which were much lower when the culture time was extended from 24 h to 48 h (REFS[84,85]). Loss of engraftment capacity is particularly problematic in inflammatory contexts, such as in patients with CGD. The impact of culture conditions on engraftment following transplantation of gene-modified HSPCs contrasts with recent reports of successful ex vivo expansion of cells from cord blood and accelerated haematological recovery in patients[86].

HSPCs react to high doses of viral vectors by activating innate immune sensors and antiviral factors that target the retroviral integration process[87]. Despite their retroviral origin, lentiviruses induced a very limited innate immune signal in HSPCs — in contrast to gammaretroviral vectors[84,88]. However, transduction of HSPCs with a lentiviral vector reportedly activated the p53 signalling pathway, which increased apoptosis, delayed proliferation (which correlated with the VCN) and decreased engraftment capacity[84]. It is noteworthy that activation of p53 signalling primarily influenced short-term repopulating stem cells; the transient inhibition of p53 signalling restored the engraftment capacity.

Over the past 5 years, numerous cell culture supplements have been tested for their ability to increase vector transduction and optimize the yield of cell products. Extensive studies have provided new information on important pathways in the early steps of lentiviral infection. Petrillo et al.[89] reported that the addition of cyclosporine and rapamycin relieves two different blocks on the early steps in lentiviral infection of HSPCs. Curiously, cyclosporine has the opposite effect in many other cell types. Heffner et al.[90] screened for bioactive small molecules and found that prostaglandin E2 boosted the lentiviral transduction of $CD34^+$ cells — confirming the data previously obtained by Zonari et al.[85]. The integration site profile was unchanged; as a result, there were no concerns that the target site selection profile was more dangerous[90]. Importantly, prostaglandin E2 also has a key role in the maintenance of HSCs[91,92] and therefore constitutes a useful agent in the ex vivo engineering of these cells. Lewis et al.[93] found that the kinase inhibitor staurosporine boosted lentiviral transduction of HSPCs (possibly by overcoming a barrier to entry). The researchers also found that staurosporine and prostaglandin E2 act through different mechanisms and that a combination of the two had a greater effect on vector transduction than either agent alone. Hauber et al.[94] assessed compounds that improved HSPC transduction with a focus on poloxamers — large, non-ionic, amphipathic molecules that are known to interact with cell membranes. The researchers found a specific polymer with good activity, which has since been marketed as LentiBOOST. These additives do not seem to interfere with HSC engraftment and differentiation, which makes them attractive as additives in cell-based manufacturing. One caveat is that increasing transduction might result in an excessively high VCN that increases the risk of genotoxicity.

Measurements of mean VCN in the drug product may even be misleading, because the VCN in the transplanted pool of HSPCs might not be the same as in HSCs with long-term repopulating ability. One aspect to be monitored closely is whether some cells in the transduction pool behave differently — for example, a small fraction might receive a very high functional multiplicity of infection and thus be exposed to a greater risk of genotoxicity.

Source of stem cells. Another important issue is the choice of the stem cell population. Knowing that only a minute fraction of infused $CD34^+$ HSPCs contributes to long-term haematopoiesis[3,95], some research groups have started to enrich the target cell population in HSCs by sorting $CD34^+CD38^-$ cells[85,96]. Beyond reducing the amount of vector required, this enrichment minimizes the differences between different sources of HSPCs (such as bone marrow versus mobilized peripheral blood HSPCs). One drawback of this strategy is delayed neutrophil recovery, which can be compensated for by either increasing the cell dose or co-transplanting uncultured, non-transduced $CD34^+CD38^+$ progenitors[85,96]. Importantly, a short cell culture (24–36 h) of sorted $CD34^+CD38^-$ HSCs in the presence of prostaglandin E2 was associated with high levels of HSC transduction[85], providing potentially optimal conditions.

Ultimately, the definitive evaluation of these transduction conditions will be determined by the quality of long-term reconstitution in patients. Analysis of the integration sites that mark each HSPC in a unique way in the initial gene therapy product enables evaluation of the clonal structure and estimation of the total number of long-term reconstituted HSC clones among the hundreds of HSPCs initially infused. These population size estimates suggest that there are at least 15–25 active stem cells per 1×10^6 $CD34^+$ cells in several disease settings[3,8], although sparse sampling means that this is probably an underestimate. Thanks to integration site tracking in gene therapy trials, recent studies have highlighted the long-term survival of T memory stem cells[97] and HSC clonal dynamics in both the early and steady-state reconstitution phases[95,98]. This constitutes a unique opportunity to map human haematopoiesis by vector marking. Comprehensive clonal analysis during the follow-up of patients receiving gene therapy potentially provides detailed data on parameters that might influence haematopoietic reconstitution, such as the conditioning regimen, the transduction conditions, the pathological context (such as in inflammatory disease states) and the source of HSPC used. For example,

it was recently demonstrated that CD34⁺ cells mobilized with G-CSF or G-CSF plus plerixafor contained significantly fewer repopulating HSCs than CD34⁺ bone marrow populations or CD34⁺ cells mobilized with plerixafor alone[79,99] — highlighting the need for further clonal comparisons of these HSPC sources with regard to long-term reconstitution in humans. This is particularly relevant for gene therapy trials in paediatric patients, in whom long-term (lifelong) reconstitution is a potential challenge.

Optimizing safety

The general patterns of retroviral integration are now well understood[1]. Gammaretroviral vectors integrate primarily within transcriptional regulatory elements (such as promoters and enhancers[100,101]), whereas lentiviral vectors integrate primarily within active transcription units[102–104]. The integration pattern varies from one retroviral genus to another, indicating that the mere exposure of different sequences in open chromatin does not account for differences in targeting[102–108]. Cell-type-specific transcription has weak but sometimes detectable effects on lentiviral vectors, which may lead to a cell-type-specific preference for integration site[109,110]. The integrase-coding region is a dominant determinant of integration site preference; this region acts by binding tethering factors[111]. Gammaretroviral vectors integrate preferentially at regulatory regions by binding cellular bromodomain and extra terminal motif proteins that, in turn, bind to acetylated histone H3 — a mark that is enriched in active promoters and enhancers[112]. The cellular integrase-binding protein lens epithelium-derived growth factor (LEDGF; also known as PC4 and SFRS1-interacting protein) targets lentiviral integration to transcription units by a tethering mechanism[113–117]. Moreover, some of the genes targeted by lentiviruses are located proximal to the nuclear pore in open chromatin[118,119].

Gammaretroviruses. The most extreme examples of cell proliferation associated with vector integration were witnessed in early gene therapy trials using gammaretroviral vectors. These vectors contained strong enhancers in the LTRs, such that integration near cancer-associated genes was linked to an increase in gene transcription and cell proliferation. In the first trial to treat SCID-X1, of 20 patients treated, 6 developed T cell acute lymphoblastic leukaemia (T-ALL) with integration near *LMO2*, *CCND2* or *BMI*[2,29,30,120]. In an early trial to treat WAS using gammaretroviral vectors, all nine treated patients developed cancers associated with vector integration near *LMO2*, *MDS1/EVI1* and other genes[45]. In DNA from the patient's blood cells, clusters of integration sites were detected near *LMO2*, *CCND2* and *MDS1/EVI1*, suggesting that integration near these genes was sufficient for clonal expansion and led to preferential recovery of those clones. Chemotherapy failed for one of the six patients with SCID-X1 who developed lymphoproliferation but was successful for all the others, who continued to benefit clinically from the gene therapy. T cell leukaemia occurred 24–68 months after gene therapy for five patients[29,30] and 15 years afterwards for one person[121]. For the most recent case, the late-onset T-ALL appeared abruptly and was characterized by an immature T cell phenotype, vector insertion 30 kb from the *LMO2* gene transcription start site and accumulation of several genetic abnormalities typically reported in T-ALL. The patient is currently finishing a course of chemotherapy and has responded well to treatment (M.C., unpublished data). This late SAE shows that the genetic network that controls growth in T cell progenitors can take many years to become dysregulated. The tumour latency correlates with the recent report of detectable mutations years before the diagnosis of acute myeloid leukaemia[122] but has never been reported in T-ALL. The late cancer onset and abrupt lymphoproliferation emphasize the difficulty of predicting pathogenic clonal expansion. In this case, the pre-leukaemic clone harbouring the *LMO2* integration site was never present as more than 2% of the peripheral blood lymphocytes. Conversely, clones in several other patients have been seen to expand transiently without the subsequent development of leukaemia[72]. The number of SAEs reported in the French trial ($n = 5$) was greater than that reported in the UK trial ($n = 1$), suggesting that small differences in the vector or the transduction protocol may have resulted in different clinical outcomes.

The ADA-SCID trials are the only gammaretrovirus-based gene therapy trials in which SAEs due to insertional mutagenesis have not been observed. An explanation for why these trials are the exception is still lacking. Given that the integration sites were similar to the vectors used in the ADA-SCID trials and those used in the trials in which oncogenic events occurred[5], one can speculate that a leukaemic clone might be counter-selected owing to its very high need for products of purine metabolism. This requirement might not be met by the ADA therapeutic gene; the malignant cells would then not be able to proliferate and thus would die.

Overall, these data show that first-generation gammaretroviral vectors should be used with great caution and that treated patients must receive rigorous long-term follow-up. The SAEs observed in the first gene therapy trials led to the development of a new generation of safer, SIN retroviral vectors that are devoid of the potent enhancer elements in the LTRs and contain a transgene cassette whose expression is driven by internal physiological promoters. These vectors have now been used in several clinical trials without any SAEs. Indeed, integration site clusters did not accumulate to the same extent near the genes of concern in a second SCID-X1 trial, which used enhancer-deleted SIN gammaretroviral vectors[31].

Lentiviruses. The first human clinical trial using lentiviral vectors with a full LTR region targeted T cells; the goal was to protect these cells from HIV infection by delivering an antisense payload[123,124]. In 2006, SIN lentiviral vectors were used for the first time to correct HSPCs in the context of ALD[69]. In both cases, the distribution of integration sites was as expected for lentiviral vectors (an elevated frequency within active transcription units), and there was no evidence of clonal expansion associated with vector integration near cancer-associated genes.

Many subsequent trials have been carried out safely using lentiviral vectors (see REFS[4,72]).

To date, more than 200 patients worldwide (TABLE 2) have been treated with new-generation SIN gammaretroviral or lentiviral gene therapy vectors, with a median follow-up of 3.6 years and no reports of SAEs. Despite the safer integration profile and the absence of potent enhancers in the LTR regions of these vectors, hundreds of millions of HSPCs are infused into each patient; therefore, caution is still warranted. Genomic insertions are

Table 2 | **HSPC-based gene therapy trials using SIN gammaretroviral and lentiviral vectors**

Disease	Trial number (phase)	Starting year; sites	Vector	Conditioning	Number of patients	Refs
Primary immune deficiencies						
SCID-X1	NCT01410019, NCT01129544 (phase I/II)	2010; France, USA	SIN *g*RV EF1*α*Sprom-IL-2Rγ	No	9	31
SCID-X1	NCT01306019 (phase I/II)	2011; USA	SIN LV EF1*α*Sprom-IL-2Rγ	Yes	8	35
WAS	NCT01515462 (phase I/II)	2010; Italy	SIN LV WASprom-WAS	Yes	7	3,170
WAS	NCT01347346, NCT01347242 (phase I/II)	2011; France, UK	SIN LV WASprom-WAS	Yes	11	4,a
WAS	NCT01410825	2011; USA	SIN LV WASprom-WAS	Yes	4	46
ADA-SCID	NCT01380990, NCT01852071, NCT02999984 (phase I/II)	2012; UK, USA	SIN LV EF1*α*Sprom-ADA	Yes	61	18,b
CGD	NCT02757911 (phase I/II)	2016; France	SIN LV Chimericprom-CYBB	Yes	2	c
CGD	NCT01855685 (phase I/II)	2013; UK, Germany, Switzerland	SIN LV Chimericprom-CYBB	Yes	4	49
CGD	NCT02234934 (phase I/II)	2015; USA	SIN LV Chimericprom-CYBB	Yes	3	49
ART-SCID	NCT03538899	2018; USA	SIN LV Artprom-DCLRE1C	Yes	NA	171
Haemoglobinopathies						
β-Thal	LG001 (phase I/II)	2007; France	SIN LV LCR-βprom-β-globin	Yes	2	72
β-Thal	NCT02151526 or HGB205 (phase I/II)	2013; France	SIN LV LCR-βprom-β-globin	Yes	4	6
β-Thal	NCT01745120 or HGB204 (phase I/II)	2013; USA, Australia, Thailand	SIN LV LCR-βprom-β-globin	Yes	18	6
β-Thal	NCT02453477 (phase I/II)	2015; Italy	SIN LV LCR-βprom-β-globin	Yes	9	172
β-Thal	NCT01639690 (phase I)	2012; USA	SIN LV LCR-βprom-β-globin	Yes	4	173
SCD	NCT02151526 or HGB205 (phase I/II)	2013; France	SIN LV LCR-βprom-β-globin	Yes	3	7,c
SCD	NCT02140554 or HGB206 (phase I)	2014; USA	SIN LV LCR-βprom-β-globin	Yes	9	78
SCD	NCT02247843 (phase I)	2014; USA	SIN LV LCR-βprom-β-globin	Yes	1	NA
SCD	NCT03282656 (phase I)	2018; USA	SIN LV LCR-βprom-BCL11A shRNA	Yes	1	174
SCD	NCT02186418-phase I/II	2014; USA, Jamaica	SIN LV LCR-βprom-β-globin	Yes	2	175
Metabolic disorders						
X-ALD	NA	2006; France	SIN LV MNDprom-ABCD1	Yes	4	69
X-ALD	NCT01896102 (phase II/III)	2013; USA, UK, France	SIN LV MNDprom-ABCD1 (Lenti-D)	Yes	17	176
MLD	NCT01560182 (phase I/II)	2010; Italy	SIN LV PGKprom-ARSA	Yes	20	8,9
MPS-I	NCT03488394	2018; Italy	SIN LV PGKprom-IDUA	Yes	1	177,178
Congenital cytopenia						
Fanconi anaemia	NCT03157804 (phase I/II)	2016; Spain	SIN LV PGKprom-FANCA	No	4	179
Fanconi anaemia	NCT01331018 (phase I)	2012; USA	SIN LV PGKprom-FANCA	Yes	2	179

The promoter and cDNA used for each vector are described. ADA, adenosine deaminase; ADA-SCID, ADA severe combined immunodeficiency; ARSA, arylsulfatase A; ART-SCID, Artemis-deficient SCID; Artprom, Artemis promoter; βprom, β-globin promoter; β-thal, β-thalassaemia; CGD, chronic granulomatous disease; Chimericprom, chimeric promoter; EF1*α*Sprom, elongation factor 1α short promoter; *g*RV, gammaretrovirus; HSPC, haematopoietic stem and progenitor cell; IL-2Rγ, interleukin-2 receptor subunit-γ; LCR, β-globin locus control region; LV, lentivirus; MLD, metachromatic leukodystrophy; MNDprom, myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer binding site substituted promoter; MPS-I, mucopolysaccharidosis type I; NA, not available; PGKprom, phosphoglycerate kinase 1 promoter; SCID-X1, X-linked SCID; shRNA, short hairpin RNA; SIN, self-inactivating; WAS, Wiskott–Aldrich syndrome; WASprom, WAS promoter; X-ALD, X-linked adrenoleukodystrophy. [a]Personal communication from A. J. Thrasher. [b]Personal communication from H. B. Gaspar. [c]M.C., unpublished data.

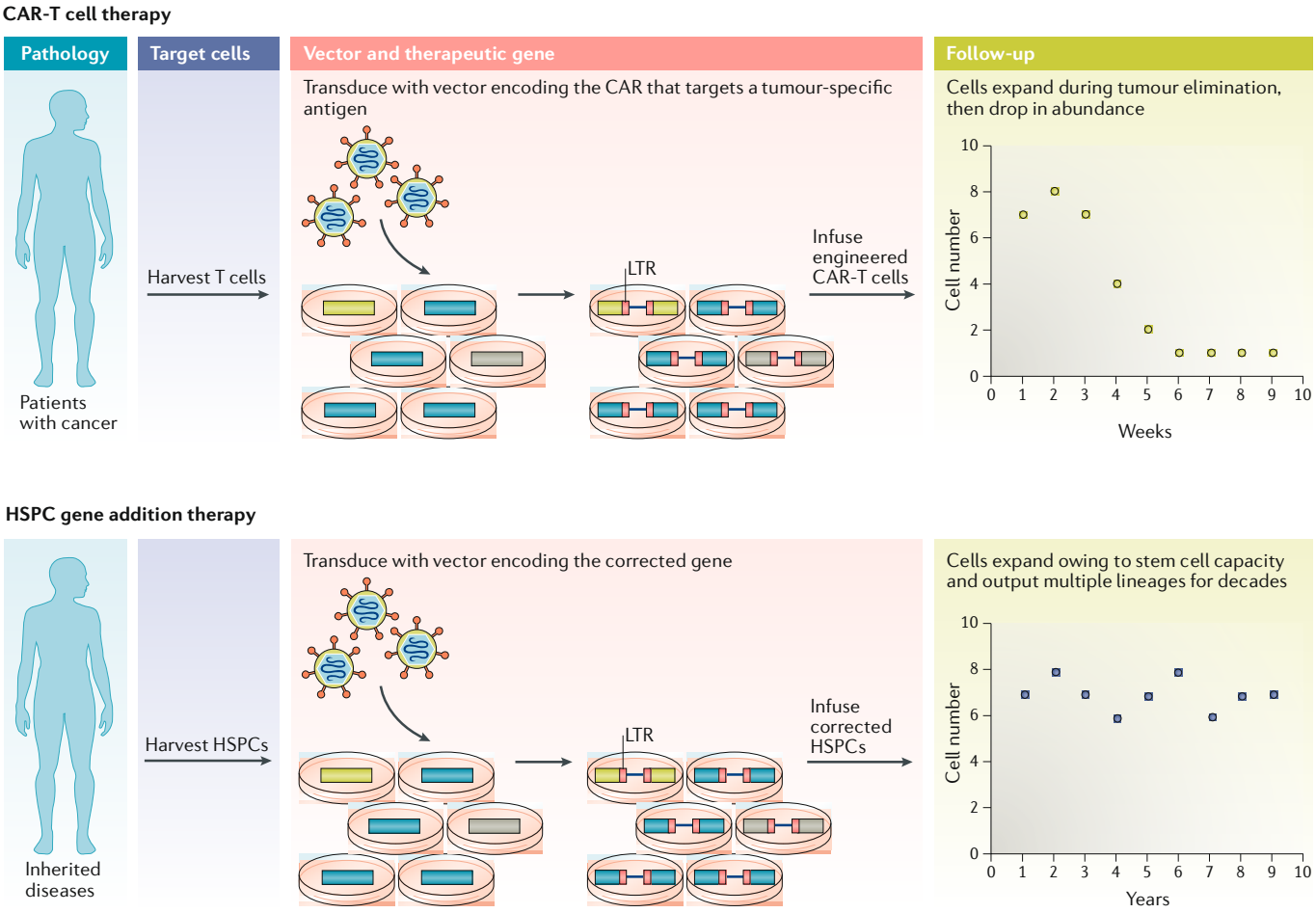

Fig. 2 | **CAR-T cell therapy versus HSPC gene addition therapy.** The clinical procedure used in chimeric antigen receptor (CAR)-T cell therapy in comparison with the haematopoietic stem and progenitor cell (HSPC) gene addition therapy is shown. The pathology of interest, the target cell, the therapeutic gene use for genetic engineering and the follow-up are indicated for each approach. LTR, long terminal repeat.

present in each of these cells, and there are still genetic mechanisms (for example, inactivation of tumour suppressor genes) that can adversely influence outcome. Lentiviral vector insertion into transcribed genes, for example, can deregulate gene expression by interfering with splicing. Aberrant splicing and chimeric transcripts can be generated through the use of constitutive and cryptic splice sites present in the vector and/or the transgene[125–127].

Some lentiviral vectors have led to prominent clonal expansions after integration within cancer-related genes, though no SAEs have resulted. As mentioned above, a longitudinal integration site analysis in the first β-thalassaemia trial[72] revealed the expansion of cells descended from a single HSC containing a vector insertion in the proto-oncogene *HMGA2*. The integration site was located in the long third intron of the gene — a site that frequently undergoes chromosomal rearrangements in lipomas and other benign tumours. This integration triggered abnormal splicing within the *HMGA2* gene, inducing the removal of the two distal exons containing binding sites for the let-7 microRNA, which acts as a negative regulator of *HMGA2* expression by promoting RNA degradation. This event — probably combined with transactivation by vector-embedded enhancer elements from the β-globin locus control region (LCR) upstream of the β-globin promoter — was associated with increased *HMGA2* expression and clonal expansion. Although other lentiviral vector trials have reported the transient appearance of clones with integration sites in or near cancer-related genes, no clinical adverse events have been linked to integration. Taken as a whole, these observations suggest that lentiviral vector integration may affect cell growth, although not as aggressively as the early gammaretroviral vectors, which had intact, potent enhancers in the LTR region.

CAR-T cell therapies. Recently, gene transduction has been used ex vivo for chimeric antigen receptor (CAR)-T cell therapy. In this method, the patient's peripheral cells are harvested and transduced with a vector encoding an engineered receptor that recognizes a surface antigen present on tumour cells (FIG. 2). The CD19 antigen has been used widely because CD19 is present on cancers of the B cell lineage, and targeting healthy B cells is clinically manageable[128–132]. A wide range of vector types has been used, including both gammaretroviral and lentiviral vectors[133]. Engineered

Box 1 | **Genome-editing strategies for haematological genetic diseases**

Several approaches for treating haematological genetic diseases have been developed by harnessing the potential of nucleases (zinc-finger nucleases, transcription activator-like effector nucleases and the CRISPR–Cas9 system) that induce double-strand breaks (DSBs) at specific genomic loci and activate cellular repair mechanisms.

Genome-editing approaches based on homology-directed repair (HDR) and a donor template containing the desired gene sequence have been investigated with a view to correcting disease-causing mutations or inserting a therapeutic transgene under the control of the endogenous promoter. Relative to gene addition strategies, these editing approaches guarantee the endogenous, physiologically regulated expression of the transgene. This is particularly important when the elements that regulate expression of the transgene have not been characterized, the gene or its regulatory elements are too large to fit into high-titre lentiviral vectors or restricted or tightly regulated transgene expression is desirable (for example, in β-haemoglobinopathies, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome[142], Artemis-deficient severe combined immunodeficiency (ART-SCID)[143], interleukin-7 receptor subunit-α-deficient SCID (IL7RA-SCID)[144] and chronic granulomatous disease (CGD)[145,146]). It is noteworthy that the use of restricted promoters to drive transgene expression with lentiviral vectors can sometimes result in near-physiological expression patterns[147,148], although several copies of the vector may be required. It is also important to note that, in autosomal recessive haematological disorders, even the genome-editing-mediated correction of only one of the two affected alleles in a reasonably high proportion of haematopoietic stem cells (HSCs) would avoid the need for multiple copies of the integrated lentiviral vector with suboptimal transgene expression. Although HDR-based approaches for correcting haematological genetic disorders are relatively efficient in haematopoietic stem and progenitor cells (HSPCs), the frequency of stably modified, repopulating HSCs rarely exceeds 10%[149–155], suggesting that stem cells might be poorly permissive to HDR (which is typical of nonquiescent cells). The inclusion of selectable markers in the donor template enables the enrichment of edited HSCs[151]; however, cell selection would be difficult to translate into the clinic because it would yield lower numbers of HSCs than conventional approaches do.

The non-homologous end-joining (NHEJ) DNA repair pathway (which is active throughout the cell cycle) has mainly been exploited to obtain permanent gene inactivation and disrupt *cis* elements that regulate gene expression via the production of small insertions and deletions after DSB generation. Given the apparent dominance of this pathway in HSCs, NHEJ-based editing strategies might be more likely to achieve clinically relevant efficiencies in HSCs than HDR-based approaches. As elevated levels of fetal haemoglobin (HbF) ameliorate the clinical phenotype of patients with β-haemoglobinopathies, NHEJ-based editing approaches have been used to downregulate the BCL11A HbF repressor[156,157] or to recreate the deletions associated with the hereditary persistence of fetal haemoglobin[158–160]. By way of an example, Chang et al. achieved an NHEJ frequency of ~50% in long-term repopulating HSCs by inactivating a BCL11A intronic enhancer[157].

Another potential advantage of genome editing over the lentiviral-based gene addition approaches is better targeting — at least in principle — via the modification of a unique, 'safe' genomic target. Lentiviral vectors insert in a semirandom fashion, mostly in intragenic regions[100], which may lead to alternative splicing, the formation of aberrant transcripts[125] and, potentially, oncogenesis[127]. However, genome editing might also give rise to nonspecific genetic modifications that could alter the function of otherwise intact genes. Furthermore, the generation of multiple on-target DSBs, simultaneous on-target and off-target DSBs or even a single on-target DSB is associated with a risk of deletions, inversions and translocations[161]. These events might impair correction of the target gene and result in the complete inactivation of a target gene (thus abrogating any beneficial residual expression that may have been present) or even elicit a long-range transcriptional effect that could constitute a first carcinogenic 'hit'. Targeted approaches that do not require the generation of DSBs (such as base editing[162] and epigenome editing[163]) may overcome this problem and can (in principle) be applied to many haematopoietic diseases. It is noteworthy that lentiviral vectors also generate DSBs, although potentially associated genomic rearrangements have not been studied.

If genome-editing strategies are to become relevant treatment options, the large-scale production of nontoxic preparations with clinical-grade reagents will be required. Given the currently high cost of conventional viral vector manufacturing, non-viral genome editing might be less expensive and thus more affordable for health-care systems. Two clinical trials of genome-editing strategies in β-thalassaemia (NCT03432364 and NCT03655678) have been approved by the US Food and Drug Administration. The results should provide information on the potential therapeutic benefits of genome editing relative to conventional lentiviral-based gene addition. Indeed, a direct comparison of genome-editing and lentiviral gene addition strategies (in terms of the efficiency of genetic modification in long-term repopulating HSCs, therapeutic gene expression, clinical efficacy and safety) will probably have to be performed for each specific disease.

cells are then expanded under conditions that favour T cell proliferation, and the modified cells are reinfused into patients. So far, no clinical adverse events associated with insertional mutagenesis in peripheral T cells have been identified, emphasizing the reduced potential for transformation of this mature cell lineage[123,134].

A recent example of clonal expansion after lentiviral vector transduction appears to have contributed to successful therapy; in this situation, CAR-T cell therapy was administered to a patient with chronic lymphocytic leukaemia[135]. This patient was treated with two infusions of CART19 cells, resulting in eventual tumour elimination. However, analysis of integration site distributions revealed a clonal expansion associated with a vector integrated within the *TET2* locus (a tumour suppressor gene involved in DNA demethylation). Extensive follow-up studies disclosed that the patient's other *TET2* allele harboured a polymorphism that diminished protein function, so the combination of the two genetic lesions led to a considerable reduction in TET2 activity. At the time when the CAR-T cell compartment was dominated by *TET2*-disrupted clones, the majority of these cells phenotypically resembled relatively undifferentiated central memory T cells — cells that are known to have greater antitumour activity[136]. These results suggest that *TET2* mutations lead to increased 'stemness' and altered T cell differentiation, which can improve therapeutic proliferative capacity. The patient is now 83 years old and has been free of leukaemia for more than 4 years since treatment. These findings suggest that adoptively transferred cells with vector integration into a specific gene may promote T cell survival, expansion and robust antitumour functions.

Future directions. Why are lentiviral vectors safer than gammaretroviral vectors? Although several forms of cancer are associated with HIV infection, the

transformed cells do not harbour integrated HIV proviruses[137], and HIV infection never results in cancer via insertional mutagenesis. This is remarkable, given that millions of people have been infected and that numerous HIV-infected cells harbour integration events. One possible explanation is that HIV does not encode strong enhancers, and high-level HIV transcription requires the viral-encoded Tat protein, which is not encoded in lentiviral vectors. In addition, the HIV-encoded Env and Vpr genes are cytotoxic, such that cells actively producing HIV are quickly killed, though these proteins are also not encoded in lentiviral vectors and thus cannot explain the lack of transformation by vectors. For HIV, clonal expansion has been reported in people with HIV, with some lymphocytes containing viral integrations within the *BACH2* or *MLK2* (also known as *MAP3K10*) gene[138,139]. Expanded clones with integrated virus within the known oncogenes *JAK2* and *SEPT9* have also been reported in a murine xenograft model of HIV infection[140]. Genotoxic effects of SIN lentiviral vectors were detected in vivo in a *Cdkn2a* knockout mouse, which is abnormally susceptible to tumours[127]. These data indicate that lentiviral vectors are associated with a low but non-zero risk of insertional mutagenesis, and this holds for the SIN gammaretroviral vectors as well. Gene therapy protocols should thus seek to correct a target cell with a single copy of the therapeutic gene, because higher VCNs increase the number of potential insertional mutations per cell and may promote aberrant clonal expansion[125,126].

In line with this hypothesis, Payen and Leboulch's group has described the properties of the BB305 β-globin lentivirus, which is capable of transducing a high proportion of haematopoietic cells with a low number of insertions per cell[141]. A codon-optimized puromycin *N*-acetyltransferase was fused to a conditional suicide gene coding for herpes simplex virus thymidine kinase, providing an additional safety mechanism because dangerous clones can be eliminated by treatment with ganciclovir. When expressed under the control of a ubiquitous promoter within the BB305 vector, viral titres and effective levels of therapeutic gene expression were maintained. Selection of the transduced HSCs was achieved via brief exposure to puromycin in the presence of blocking agents for multidrug resistance protein 1, suggesting that the procedure is suitable for clinical testing.

Conclusions

The infusion of HSPCs that are genetically modified with retroviral vectors has proved its therapeutic potential for several very severe, life-threatening diseases. Gene therapy can be further improved by leveraging recent discoveries in HSPC and viral biology and new developments in vector design and transduction.

Despite all this progress, the commercialization of this HSC gene therapy remains extremely challenging. The biomanufacturing of genetically modified HSPCs has not changed much over the past two decades. These and other issues impede the broader dissemination of the gene therapy approach. The implementation of decentralized manufacturing (after the requisite technological and regulatory changes) would increase the number of centres able to administer this therapy and thus ease the currently unacceptable burden of travel placed on potential patients and their families.

The recent, extensive developments in vector design, transduction efficiency and HSPC isolation and processing have already proved the effectiveness of the gene therapy approach and have allowed complicated diseases to be successfully treated. On the basis of ongoing international research in this field, we can expect to see an increase in the number of haematological and non-haematological diseases treated by HSPC-based gene therapy in the coming years. Lentiviral-based gene addition therapy constitutes an essential therapeutic approach for numerous haematological diseases. Pioneering trials using gene-editing approaches have also started recently (BOX 1). Gene editing will probably require considerable optimization if it is to achieve the same level of effectiveness as gene-addition-based strategies and will require further safety evaluations. In the meantime, the numerous breakthroughs accomplished by the field should allow a continuously growing number of inherited diseases to benefit from gene addition therapy.

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Competing interests
The authors declare no competing interests.