

# HEMATOPOIETIC STEM CELL GENE THERAPY FOR INHERITED MONOGENIC DISEASES AND ITS IMPLICATIONS FOR FUTURE GENE THERAPY TRIALS IN TURKEY

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**Abstract:** Stem cell therapy offers a great advantage for the development of new treatments in the field of regenerative and restorative medicine. However, the use of stem cell therapies and their clinical indications can even be further improved using genetic modification of the cells. Due to the high level of consanguineous marriages in Turkey, the country suffers from an increased frequency of inborn genetically inherited diseases. Treatment of these diseases is difficult, since 1) diagnosis is often delayed in rural areas, 2) distance to specialized centers may be considerable, 3) treatment may require frequent hospital visits and 4) treatment procedures are often both invasive and expensive.

Here, we discuss the current status of gene therapy of hematopoietic stem cells (HSCs) for rare, inherited monogenic diseases and the advantages to use these cells as an alternative treatment option for patients in Turkey. We discuss results of clinical trials using retroviral and lentiviral gene therapy for the treatment of immune deficiencies, hemoglobinopathies and several enzyme deficiencies, new developments in the field of the HSC gene therapy to improve safety and efficacy and recommendations for the future.

**Key words:** Gene Therapy, hematopoietic stem cells, lentiviral vectors, biosafety, inherited monogenic diseases.

**Özet:** Kök hücre tedavisi rejeneratif tıp ve restoratif tıp alanlarında yeni tedavilerin geliştirilmesi için büyük avantaj sağlamaktadır. Böylelikle, kök hücre tedavilerinin kullanımı ve bunların klinik endikasyonları hücrelerin genetik modifikasyonu ile geliştirilebilmektedir. Türkiye'deki akraba evlilik oranının fazla olmasından dolayı, yenidoğan genetik kalıtsal hastalıkların insidansı artmaktadır ve bu durum bir sorun teşkil etmektedir. Bu hastalıkların tedavi edilmesi; 1) kırsal bölgelerde hastalığın sıklıkla geç teşhisi, 2) Özel merkezlerin uzak olması, 3) Tedavilerin sık hastane kontrolü gerektirmesi, 4) Tedavi prosedürlerinin hem pahalı hem de invazif olması nedenleriyle zordur.

Bu makalede nadir kalıtsal monogenik hastalıklar için hematopoetik kök hücre (HKH) gen tedavisinin güncel durumları ve Türkiye'deki hastalar için alternatif bir tedavi seçeneği olarak kullanımının avantajları tartışılacaktır. İmmün yetmezlikler, hemoglobinopatiler, birçok enzim eksikliklerinde retroviral ve lentiviral gen tedavi klinik çalışma sonuçları, HKH gen tedavisi alanındaki yeni gelişmeler, güvenlik ve etkinliğin artırılması ve gelecekteki öneriler tartışılacaktır.

## Introduction

### Current treatment strategies for inherited monogenic diseases

The rationale for stem cell gene therapy for inherited diseases is to provide the genome with a healthy copy of the gene as an addition to or as a replacement of the mutated gene in order to develop permanently curative treatment options for inherited monogenic diseases. Many hematopoietic diseases and enzyme deficiencies can be currently treated with hematopoietic stem cell (HSC) transplantation (Biffi 2017, Majhail *et al.* 2015, Ringden *et al.* 2018, Wynn 2011). In addition, Enzyme

Replacement Therapy (ERT) has been developed for some of the enzyme deficiencies/storage diseases, such as Fabry disease, Gaucher disease, Pompe disease, Lysosomal acid lipase deficiency, Mucopolysaccharidosis (MPS-I: Hurler Syndrome; MPS-II: Hunter Syndrome; MPS-IV: Morquio Syndrome; MPS-VI: Maroteaux-Lamy syndrome), as well as for severe combined immunodeficiency (SCID) caused by adenosine deaminase deficiency (ADA-SCID) (Beck 2018, Gaspar *et al.* 2009). However, not all patients have an available matched donor, and even if a donor is available, the HSC transplantation will fail when



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expression levels of the enzyme in HSCs are too low to expect benefit, or may result in graft-versus-host-disease (GvHD) when cells derived from the donor induce immunological damage of tissues/organs of the patient. In addition, ERT often involves life-long weekly-monthly treatments requiring frequent (out-patient clinic) hospital admissions and effects of ERT typically decline in time due to immune responses against the drugs, resulting in even more frequently needed infusions. Moreover, the inability of enzymes to efficiently cross the blood-brain barrier (BBB) limits the efficacy of both HSC transplantation and ERT in enzyme deficiencies that affect the central nervous system (CNS).

#### Development of novel treatments for rare diseases

Rare or orphan diseases are diseases which affect fewer than 200,000 people (US definition) or less than 5 individuals per 10,000 of the population (EU definition) and as a result are not the focus of interest of the pharmaceutical industry because of the little financial incentive to develop new medications (Sharma *et al.* 2010). Approximately 70% of about 6,000-8,000 diseases which are considered rare are genetic in their origins. To encourage pharmaceutical companies to invest in orphan drug development, the US introduced the US Orphan Drug Act in 1983 to provide suitable tax initiatives on clinical trials and 7 years of marketing exclusivity for drugs developed for conditions that occur only rarely in the US (Shah 2006). Since then, over 250 orphan drugs have been approved by the US Food and Drug Administration (FDA) and in its footsteps, a number of other countries introduced similar legislation (Singapore in 1991, Japan in 1993 and Australia in 1998) (Shah 2006). In 2000, the European Parliament and Council Regulation (EC) adopted the orphan medicinal products legislation with regulations No 141/2000 (EC No 141/2000) and No 847/2000 (EC No 847/2000). The EMEA (now EMA), through its Committee for orphan medicinal products (COMP) is responsible for reviewing designation applications. During the first five years of implementation (from April 2000 to April 2005), 458 applications for orphan designation were submitted, resulting in 268 products being designated for more than 200 different rare conditions (EC No 141/2000). The predominant therapeutic areas covered were cancer (36%), metabolic disorders (21%), immunology (11%), and cardiovascular and respiratory disorders (12%) (EC No 141/2000). Of these, 54% of the medicinal products designated had potential for pediatric use, 11% solely for pediatric and 43% for both adult and pediatric use (EC No 141/2000). Unfortunately, research funds barely cover the costs of the scientific community and development of new innovative medicinal products is expensive. It is estimated that the average cost of developing a new drug ranges from 400 to 800 million USD depending on the therapeutic class of the drug (DiMasi *et al.* 2003). Commercially, the costs for the development of a drug for an orphan disease often do not outweigh the expected returns. As a result, prices may be high and access may

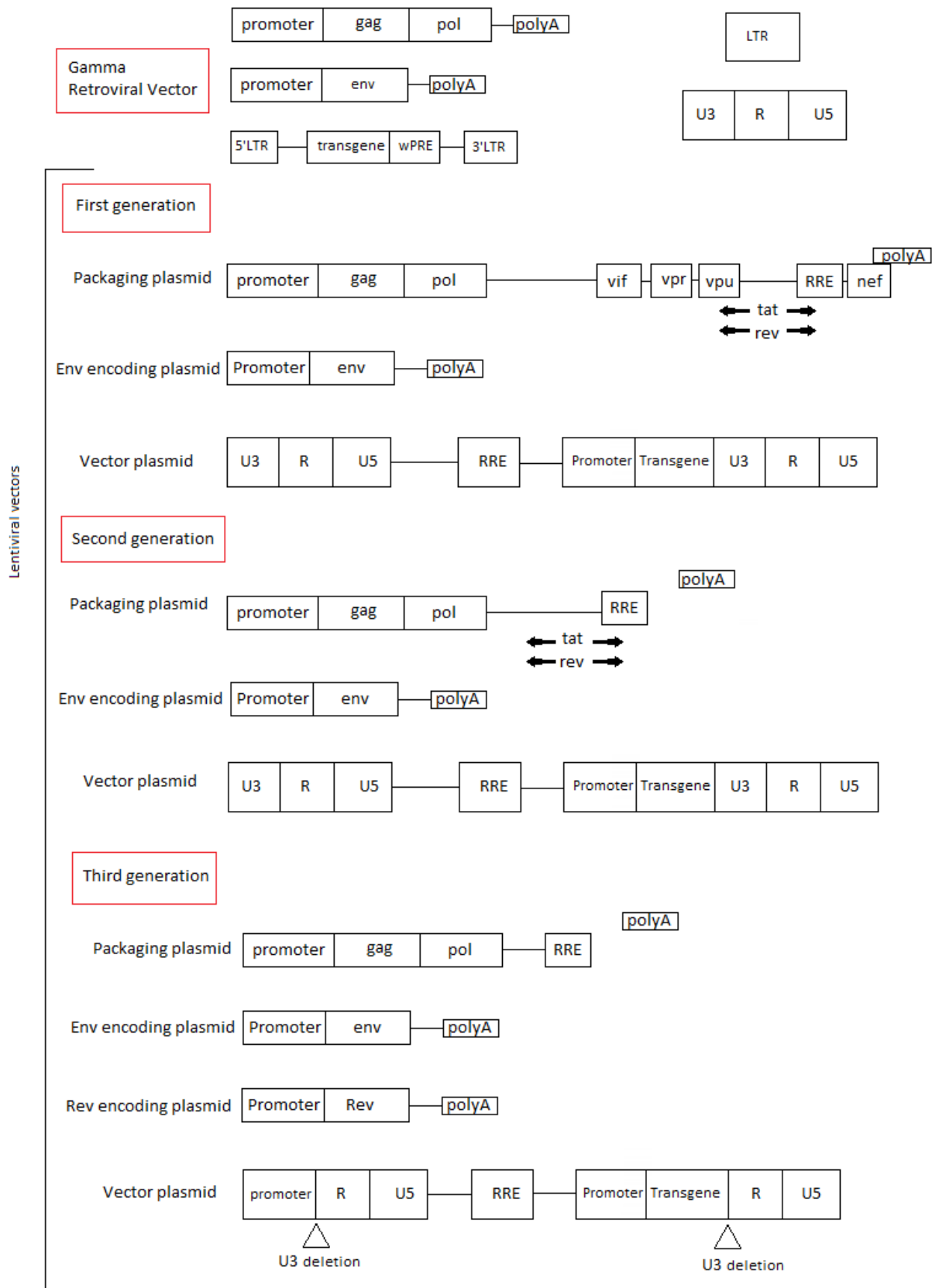
vary from country to country and depend on the availability of health insurance or governmental reimbursements.

#### Gene therapy for inherited monogenic diseases

After an initial period of low success rates with unforeseen severe adverse effects and poor public understanding resulted in the (temporarily) seizing of ongoing clinical trials, gene therapy is now becoming more and more attractive as a treatment option for rare, inherited monogenic diseases. Not only because the treatment is (intended to be) curative and a single treatment should be sufficient, but also because the costs of a single treatment would be significantly lower than the costs of life-long symptomatic treatment. The use of genetically modified autologous HSCs to treat monogenic diseases is based on the assumption that 1) the disease itself can be treated by the transfer of healthy HSCs because the hematopoietic stem cell and/or its progeny are affected, e.g. primary immune deficiencies (PID) or diseases involving hemoglobin synthesis (hemoglobinopathy), 2) addition of a single normal copy of the mutated gene is sufficient to correct the deficiency or alleviate its symptoms or 3) overexpression of the gene in hematopoietic cells results in sufficient levels of the missing protein, resulting in cross-correction of affected cells and clearance of the accumulated substrate, e.g. enzyme deficiencies. In addition, gene therapy in mitotic cells, such as HSCs, lymphoid progenitor cells and mature lymphocytes requires the use of a vector system such as retrovirus or lentivirus which are able to integrate in the host genome. This property ensures replication of the therapeutic transgene during cell division and stable transmission to its progeny. Although a substantial part of gene therapy studies and clinical trials (64.5%) focuses on the treatment of cancer, here we will address only gene therapy for inherited monogenic diseases and its foreseen use in the Turkish population. Discussion of gene therapy for multifactorial diseases or malignancies is beyond the scope of this article and has been reviewed recently by Sanlioglu (2016).

#### Development of retroviral and lentiviral vectors for HSC gene therapy

The gammaretroviral genome ( $\gamma$ RV) consists of a ssRNA and is approximately 7-10 kb in length. Retroviral vectors can stably integrate into the host genome using the enzymes reverse transcriptase (RT), which turns ssRNA into ssDNA and integrase (IN), which facilitates integration into the host genome. Gammaretroviral vector proviral DNA consists of a 5' and a 3' long terminal repeat (LTR), consisting of a U3, R and a U5 region, as well as open reading frames (ORF) for structural (Naldini *et al.* 1996), replication (pol) (Puthenveetil *et al.* 2004) and envelope proteins (Maetzig *et al.* 2011). The first retroviral constructs have been developed almost 40 years ago and were initially based on murine leukemia virus (MLV) (Shimotohno & Temin 1981, Wei *et al.* 1981).



**Fig. 1.** Development of different generations of retroviral and lentiviral vectors. To generate second ( $\Delta Vpr$ , Vif, Vpu, Nef) and third (also  $\Delta Tat$ ) generation vector systems, genes not required for transduction were deleted. To improve safety, the *rev* gene was deleted from the packaging construct and the promoter-enhancer sequences in the 5'LTR U3 region of the integrated transgene were deleted, creating self-inactivating (SIN) lentiviral vectors.

**Table 1.** Design and safety features of different retroviral and lentiviral constructs.

Vectors	Plasmids	Features	Insertional mutagenesis	Ref
$\gamma$ RV (MLV)	1) gag, pol, env; 2) vector genome and transgene	genome integration, restricted to dividing cells, RCR	+	(Maetzig <i>et al.</i> 2011)
First generation LV	1) gag, pol, rev, vpi, vpu, vpr, nef; 2) env; 3) vector genome and transgene	genome integration, dividing and non-dividing cells, RCL	+	(Naldini <i>et al.</i> 1996)
Second generation LV	1) gag, pol, rev, $\Delta$ vpr; 2) env; 3) vector genome and transgene	genome integration, dividing and non-dividing cells, low RCL	+	(Zufferey <i>et al.</i> 1997)
Third generation SIN-LV	1) gag, pol; 2) env; 3) rev; 4) vector genome and transgene	self-inactivating, genome integration, dividing and non-dividing cells, very low RCL	+/-	(Dull <i>et al.</i> 1998)
Fourth generation SIN-LTR1, Lenti-X	1) gag; 2) $\Delta$ vpr, pol; 3) tat, rev; 4) env; 5) vector genome and transgene	Genome integration, dividing and non-dividing cells, very low RCL	+/-	(Berkhout 2017, Vink <i>et al.</i> 2017)
NILV	1) gag, $\Delta$ pol (deletion of integrase); 2) env; 3) vector genome and transgene	No genome integration, dividing and non-dividing cells	-	(Banasik & McCray 2010, Shaw & Cornetta 2014, Wanisch & Yanez-Munoz 2009)

Since retroviruses use receptor binding to transfer their genetic material into specific somatic cells, this mechanism can be modified and used to transfer transgenes for therapeutic intervention into specific target cells, eg. HSCs and treat monogenic diseases. To prevent the generation of replication competent retrovirus (RCR) in genetically modified cells, the genes for Gag/Pol and Env need to be separated using a split packaging design. The resulting retroviral vector contains the packaging signal ( $\psi$ ), the primer binding site (PBS) and the long terminal repeats (LTR), but carries the transgene instead of the Gag/Pol and Env genes, which are expressed by separate plasmids. After integration of the retroviral DNA into the host cell chromosomal DNA, the proviral DNA is replicated during the cell cycle, and subsequently passed to the cell's progeny (Buchsacher & Wong-Staal 2000, Schambach *et al.* 2009, Sinn *et al.* 2005). However,  $\gamma$ RV vectors can only infect dividing cells and permanent insertion has been shown to be associated with a risk for insertional mutagenesis (Hacein-Bey-Abina *et al.* 2003, Temin 1990).

In the 1990s, Naldini *et al.* (1996) developed lentiviral vectors based on the Human immunodeficiency virus-1 (HIV-1). Lentivirus (LV) has the same basic biological features as  $\gamma$ RV (Nisole & Saib 2004). However, LV vectors have the additional ability to transfer genetic material to both dividing and non-dividing cells and are considered safer than  $\gamma$ RV (Lewis & Emerman 1994, Schambach *et al.* 2013). Lentiviruses, when compared to oncogenic  $\gamma$ RV, have a more complex genome and have two regulatory genes, tat and rev, which are indispensable for viral replication

and four unnecessary accessory genes, vif, vpr, vpu and nef, which are key for *in vivo* replication and pathogenesis of HIV-1 but not required for *in vitro* viral expansion or transduction.

The first generation LV vectors developed by Naldini *et al.* (1996) consisted of a three plasmid system. The first plasmid contained the genes for gag, pol, tat and rev, as well as the accessory genes and the packaging signal, the second plasmid encoded the heterologous env protein, which determines vector tropism and the third plasmid included the transgene or gene of interest (GOI) (Naldini *et al.* 1996). To generate second ( $\Delta$ Vpr, Vif, Vpu, Nef) (Dull *et al.* 1998) and third (also  $\Delta$ Tat) (Miyoshi *et al.* 1999) generation vector systems, genes not required for transduction were deleted and to increase safety, the rev gene was also deleted from the packaging construct, making expression of gag and pol strictly dependent on Rev complementation *in trans* on a separate plasmid (Dull *et al.* 1998). Therefore, in third generation LV vectors, generation of RCL requires two recombination events. Furthermore, as an additional safety feature, the promoter-enhancer sequences in the 5'LTR U3 region of the integrated transgene were deleted, creating self-inactivating (SIN) lentiviral vectors with a decreased risk for generation of replication competent lentivirus (RCL) (Zufferey *et al.* 1997). However, third generation LV vector genomic RNA still requires sequences to partially overlap wild-type HIV-1 gag and env genes for packaging into vector particles. To circumvent this problem and further decrease the total HIV-1 content in the LV vectors, a fourth generation LTR1 vector has been designed to prevent potential transfer of HIV-1 packaging sequences

to host cells by building a system wherein reverse-transcription results in single strand transfer, instead of the usual two (Berkhout 2017, Vink *et al.* 2017). Here, the lentiviral vector was further modified by placing several essential RNA signals (PBS- $\Psi$ -RRE) outside the viral backbone and downstream of the 3'LTR (Berkhout 2017, Vink *et al.* 2017). Alternatively, in the Lenti-X packaging system the *gag* and *pol* genes are further separated onto two plasmids, requiring at least three low-frequency recombination events to generate RCL. Although retroviral vectors were shown to have a preference for integrations near specific proto-oncogenes (*LMO2*, *CCND2*, *MSD1-EVII*, *PRDM16*, *SETBP1*) (Deichmann *et al.* 2007, Howe *et al.* 2008, Schwarzwaelder *et al.* 2007), SIN-LV vectors, which are considered less genotoxic than the  $\gamma$ RV vectors, also carry non-negligible risks of insertional transformation (Modlich *et al.* 2009). Therefore, the last major advance has been the development of non-integrating LV (NILV) or integration deficient lentiviral (IDLV) vectors. This design aims to remove genome integration and eliminate any risk of recombination and insertional mutagenesis. NILV vectors can be made by inducing mutations in *pol* that alter the integrase protein and affect its function, or alternatively by deletion of a 12 bp fraction of the 5'LTR U3 unit and an 11 bp fraction of the 3'LTR U5 resulting in mutations in the integrase DNA attachment site (LTR *att* sites) (Banasik & McCray 2010). Despite these modifications, the vectors maintain the ability to enter target cells, perform reverse transcription, transport the pre-integration complex (Hacein-Bey Abina *et al.*) into the nucleus and efficiently express their transgene product. The modifications also do not affect the capacity of NILV vectors to efficiently infect both dividing and non-dividing cells (Shaw & Cornetta 2014, Wanisch & Yanez-Munoz 2009). The design and safety features of different RV and LV vectors are summarized in Table 1 and the most commonly used vector constructs are depicted in Fig. 1.

### Status quo of ex vivo gene therapy for inherited monogenic diseases

#### Primary immune deficiencies

Primary immune deficiencies (PIDs) are the result of mutations in genes required during development of specific leukocytes, i.e. T, B and/or NK cells. Until recently, allogeneic hematopoietic stem cell transplantation (HSCT) was the only curative treatment option for PID patients. However, the outcome of HSCT in SCID depends on many factors including histocompatibility, conditioning regimen, manipulation of the graft, and T-cell depletion (Friedrich & Honig, 2010). In addition, allogeneic HSCT is often complicated by severe side effects resulting from both the conditioning regimen and graft versus host disease (GvHD). In cases where a suitable donor is unavailable, gene therapy offers the advantage of using the patient's own cells (autologous), thus preventing the immunologic complications related to GvHD. Moreover, the selective

growth advantage of the genetically modified immune competent cells allows for minimal or no conditioning (EBMT/ESID guidelines for haematopoietic stem cell transplantation for primary immunodeficiencies 2017). Recent clinical trials using gene therapy have led to (partial) immune restoration in patients with X-linked SCID (X-SCID), adenosine deaminase (ADA)-deficient SCID, Wiskott-Aldrich syndrome (WAS) and chronic granulomatous disease (CGD).

The most common PID is X-linked SCID. The disease is the result of a mutation in the *IL2RG* gene encoding the common gamma chain ( $\gamma$ c) cytokine receptor subunit. This subunit is shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Abnormal signaling through these receptors results in disruption of the development of T- and NK-cells and B-cells, although present, cannot function properly due to the absence of T-cells. Between 1999 and 2006, 20 children for whom an HLA-compatible donor was not available, were treated in two gene therapy trials for X-SCID in Hôpital Necker, Paris (Cavazzana-Calvo *et al.* 2000, Hacein-Bey-Abina *et al.* 2002) and Great Ormond Street Hospital (GOSH, London) (Gaspar *et al.* 2004). The vectors were based on the use of  $\gamma$ RV constructs in which the therapeutic gene, *IL2R $\gamma$* , was placed under the transcriptional control of the LTR, with the use of either an amphotropic envelope (Cavazzana-Calvo *et al.* 2000) or the gibbon ape leukemia virus (GALV) envelope (Gaspar *et al.* 2004). All children received *ex vivo* transduced CD34+ cells without preconditioning. Doses ranged from 5 to 20x10<sup>6</sup> CD34+  $\gamma$ c+ cells/kg weight. Between 31-68 months after infusion of the gene modified cells, 4 of the 10 patients from the Paris trial (Hacein-Bey-Abina *et al.* 2008) and 1 of the 10 patients in the London trial (Howe *et al.* 2008) developed a T-cell lymphoproliferative disorder. In the Paris trial, 2 patients had blast cells with activating vector insertions near the LIM domain-only 2 (*LMO2*) protooncogene (Hacein-Bey-Abina *et al.* 2003), 1 patient had blast cells with an integrated vector near *LMO2* and a second integrated vector near the proto-oncogene *BM11* and in the following patient, blast cells contained an integrated vector near a third proto-oncogene, *CCND2* (Hacein-Bey-Abina *et al.* 2008). The patient in the London trial developed T-ALL, and integration of the vector was found in an antisense orientation 35 kb upstream of *LMO2* (Howe *et al.* 2008). Although the role of *LMO2* alone in oncogenesis has been shown in primary T-cell leukemia (Royer-Pokora *et al.* 1991) and hematopoietic tumors in mice (Dave *et al.* 2004), leukemogenesis in these patients was likely the result of overexpression of *LMO2*, followed by the occurrence of other genetic abnormalities unrelated to vector insertion, including a gain-of-function mutation in *NOTCH1*, deletion of the tumor suppressor gene locus cyclin-dependent kinase 2A (*CDKN2A*), and translocation of the TCR- $\beta$  region to the *STIL-TAL1* locus (Hacein-Bey-Abina *et al.* 2008, Howe *et al.* 2008). Chemotherapy led to sustained remission in 4 of the 5 patients and was associated with restoration of polyclonal gene-corrected T-cell populations with a fully diverse T-cell repertoire.

The remaining subject passed away from refractory leukemia despite chemotherapy. As a result, a total of 19 out of 20 patients benefited from the therapeutic gene transfer (Hacein-Bey-Abina *et al.* 2008, Howe *et al.* 2008) and up to 10 years later, 17 were alive and maintained (nearly) full correction of the T-cell immunodeficiency (Cavazzana-Calvo *et al.* 2000, Hacein-Bey-Abina *et al.* 2002, Howe *et al.* 2008). Gene therapy at NIH in 3 children from 10 to 14 years of age, receiving up to  $30 \times 10^6$  CD34+  $\gamma c+$  cells/kg was moderately successful (Chinen *et al.* 2007), whereas treatment of two older patients (20 years old) and receiving  $0.8 \times 10^6$  and  $4.5 \times 10^6$  CD34+  $\gamma c+$  cells/kg, respectively, completely failed to produce lasting recovery of T-cell immunity (Thrasher *et al.* 2005). Increasing age, as well as a clinical history of chronic infection and GvHD negatively affect the chances of the gene transfer treatment to restore effective thymopoiesis. In addition, thymus hypoplasia as a result of prolonged absence of interaction between T-precursor cells and thymic epithelium can become irreversible. Therefore, it is strongly recommended that gene therapy in eligible SCID patients should be done as soon as possible after diagnosis (Aiuti & Roncarolo 2009).

Adenosine deaminase (ADA) is an essential enzyme of the purine metabolism, which catalyzes the deamination of adenosine and deoxyadenosine in the purine catabolic pathway after DNA breakdown and is expressed in all tissues of the body (Blackburn & Kellems 2005). ADA deficiency is a fatal, autosomal recessive disease and results in the intracellular accumulation of its metabolic substrates, dAXP and adenosine, causing toxicity not only in lymphoid progenitor cells, but also in bone, brain, lungs, liver and epithelia. The condition is particularly severe as the development of T, B and NK-cells is affected, resulting in defects in both cell-mediated and humoral immunity (Blackburn & Kellems 2005). The disease can be treated by HLA-identical HSCT (Antoine *et al.* 2003), with current survival rates up to 90% if treated at an early time point (Gaspar *et al.* 2009), but the use of alternative donors, such as matched unrelated donors (MUD) or umbilical cord blood (UCB), is associated with a high risk of (treatment-related) death or lack of engraftment (Gaspar 2010). Alternatively, enzyme replacement therapy (ERT) with Poly-ethylene glycol-modified ADA (Peg-ADA) can be started (Hershfield 1995). However, comparison of HSCT and ERT showed that where HSCT results in a constant peripheral T-cell number and diversification of the T-cell repertoire and an increase in B-cells, ERT results in a slowly progressive narrowing of the T-cell repertoire and a decrease in CD19+ lymphocytes in comparison to age-matched children (Serana *et al.* 2010). Initially, ADA-SCID patients were treated with retrovirally transduced peripheral blood lymphocytes (PBL) or autologous UCB (Kohn *et al.* 1998). Most patients displayed long-term persistence of transduced T-cells in the circulation, in the absence of toxicity, but due to low efficiency of gene transfer and engraftment, correction of the immunologic and metabolic defects was insufficient and all patients

continued to receive ERT (Aiuti *et al.* 2002, Blaese *et al.* 1995, Bordignon *et al.* 1995, Kohn *et al.* 1998). Subsequently, more than 30 ADA-SCID patients have been treated with gene therapy in different transplantation centers worldwide (Cappelli & Aiuti 2010, Ferrua *et al.* 2010a). 15 patients were treated with retrovirally transduced autologous HSC in HSR-TIGET, Italy (Aiuti *et al.* 2009, Aiuti & Roncarolo, 2009, Aiuti *et al.* 2002), 10 in CHLA-NIH, USA (Engel *et al.* 2007, Sokolic *et al.* 2008), 5 in GOSH, UK (Gaspar *et al.* 2009, Gaspar *et al.* 2006), and 2 in Hokkaido, Japan (Otsu *et al.* 2006). The patients from the Italy trial displayed progressive immune reconstitution and long-term multilineage engraftment and were able to discontinue ERT treatment in 13 of the 15 cases (Aiuti & Roncarolo 2009). In contrast to the gene therapy trials for X-SCID, no cases of leukemia related to insertional mutagenesis have been reported in patients treated for ADA-SCID (Aiuti *et al.* 2007), despite the use of a similar  $\gamma RV$  vector and the observation of a similar frequency of integration near *LMO2* and other proto-oncogenes (Aiuti *et al.* 2007). These differences have been attributed to the use of different internal promoters and the transgene itself. These positive results have resulted in the approval of Strimvelis, the first gene therapy drug for treatment of children with ADA-SCID, in 2016 by the European Commission (Aiuti *et al.* 2017, European Medicines Agency 2016).

X-linked CGD is an inherited primary immunodeficiency disease, due to a defect in the *gp91phox* gene that encodes a NADPH oxidase transmembrane protein. CGD affects phagocytes resulting in defective intracellular killing (Kang *et al.* 2011), causing recurrent bacterial and fungal infections. The first gene therapy trials for CGD took place at the NIH in the 1990s using  $\gamma RV$  vectors (Malech *et al.* 1997). However, these gene therapy trials failed to sustain long-term engraftment of the retrovirally transduced CD34+ cells (Grez *et al.* 2011). In order to improve engraftment of transduced CD34+ cells, low dose non-myeloablative conditioning was used in following studies in two adults and two children. This resulted in improved engraftment and temporary clinical benefit. However, due to insertional activation of the proto-oncogene *MDS1-EVII* and transgene silencing, this clinical benefit was eventually lost and all patients developed myelodysplastic syndrome (MDS) (Ott *et al.* 2006, Siler *et al.* 2015, Stein *et al.* 2010). In total, 5 clinical trials were performed using  $\gamma RV$  vectors and cell engraftment progressively decreased with time in all 12 patients, with several patients developing MDS. In contrast to the gene therapy of other immune deficiencies, corrected HSCs did not appear to have a selective growth advantage. Furthermore, it has been suggested that the constitutive expression of *gp91phox* in HSCs may have led to overproduction of ROS, causing cellular toxicity and loss of the genetically corrected stem cells over time (Arnold & Heimall 2017). Current gene therapy trials for CGD make use of codon-optimized third generation SIN-LV vectors to decrease the risk for insertional mutagenesis (Keller *et al.* 2018),

and specific internal promoters designed to allow transgene expression restricted to the myeloid lineage only using a chimeric promoter (Santilli *et al.* 2011) or a myeloid specific promoter (Chiriaco *et al.* 2014).

Wiskott-Aldrich Syndrome (WAS) is a disorder caused by a mutation in the *WAS* gene that encodes WASp, a regulator protein of the main actin cytoskeleton and resulting in eczema, microtrombopenia, infections and autoimmunity in patients. In the first clinical trial for WAS a  $\gamma$ RV vector was used, which resulted in correction of the functional defect. However, 7 of the 9 treated patients developed leukemia, because of integrations in the *LMO2*, *MDS1/EVII*, *MNI* proto-oncogenes (Ghosh & Gaspar 2017). The use of a SIN-LV-WASp vector to treat an adult WAS patient and 10 children showed promising results with rapid engraftment and sustained clinical improvement in the absence of insertional mutagenesis, showing that LV-based gene therapy may be developed as an alternative treatment for WAS (Hacein-Bey Abina *et al.* 2015, Morris *et al.* 2017).

### Hemoglobinopathies

$\beta$ -Thalassemia ( $\beta$ -Thal) and Sickle Cell Disease (SCD) are the most common inherited monogenic disorders throughout the world, with more than 300,000 affected neonates born annually. Hemoglobinopathies are particularly common in Asian and Mediterranean countries, one of which is Turkey (Higgs *et al.* 2012). Especially, the incidence of  $\beta$ -Thal in the Marmara region (up to 11.7%) and southern parts (up to 13.1%), and SCD (up to 47%) in Çukurova region of Turkey are very high (Kilinc 2006). According to a survey conducted by the Ministry of Health and hemoglobinopathy council in 2006, 5000  $\beta$ -Thal cases have been reported in Turkey, whereas the prevalence of SCD in Turkey is estimated to be 0.03-0.06%. Both  $\beta$ -Thal and SCD are recessively inherited hemoglobinopathies characterized by a mutation in the hemoglobin  $\beta$  (HBB) gene. In SCD, a point mutation results in the formation of hemoglobin S (HbS), which polymerizes in the deoxygenated state, resulting in red blood cell sickling. Most patients suffer from chronic hemolytic anemia, acute and chronic pain, pulmonary and renal failure, cardiovascular disease and a cognitive decline.  $\beta$ -Thal results in reduced or abrogated  $\beta$ -hemoglobin production and ineffective erythropoiesis. Thalassemia major patients are transfusion dependent and suffer from severe hemolytic anemia, iron overload, hepatosplenomegaly, cardiomyopathy, endocrine disorders and skeletal abnormalities due to bone marrow expansion. The only current treatment of hemoglobinopathies is allogenic HSCT from an HLA-matched donor. Genetic correction of autologous HSCs could serve as an alternative treatment option for hemoglobinopathies in the absence of a suitable donor.

In order to increase the safety profile of LV vectors and improve expression of the Hb transgene, Puthenveetil *et al.* (2004) designed a 1.2 kb cHS4 insulator sequence, which blocks enhancer activity, preventing activation of

nearby oncogenes, and reduces silencing of the transgene by heterochromatin. Further modifications of this insulator were used in following trials. In 2007, a single patient was infused with autologous CD34<sup>+</sup> HSCs transduced with the HPV569 SIN-LV vector containing two copies of the cHS4 chromatin insulator in the U3 region and encoding a mutated adult  $\beta$ -globin ( $\beta^{A(T87Q)}$ ) with anti-sickling properties (Cavazzana-Calvo *et al.* 2010, Negre *et al.* 2015). Three years after transplantation, the patient was transfusion independent with a stable level of 8-9 g/dL Hb. A subsequent trial used an optimized version of the LV construct (Negre *et al.* 2015), without the cHS4 insulator and included a total of 18 patients with  $\beta$ -Thal and 4 patients with SCD BB305. All patients (n=22) showed a highly polyclonal integration profile, with no clonal dominance, had stable Hb levels and became transfusion-independent within 12 months after GT (n=15) or required significantly less red blood cell transfusions (n=7) (Cavazzana 2016, Thompson *et al.* 2016). The gene therapy trial resulted in stable hematopoietic reconstitution, was well tolerated and did not cause severe adverse events. A clinical trial in 2012 in four patients using the wild-type  $\beta$ -globin transgene resulted in limited gene transfer and lack of efficacy (Mansilla-Soto *et al.* 2016). In 2015, a clinical trial phase I/II was started in Italy for transfusion dependent patients. HSCs were transduced with the GLOBE SIN-LV vector, which expresses the wild-type  $\beta$ -globin transgene under the control of the  $\beta$ -globin promoter (Miccio *et al.* 2008, Roselli *et al.* 2010). In 2017, seven patients (3 adults and 4 children) with different genotypes had been enrolled and were treated with GLOBE-SIN-LV transduced CD34<sup>+</sup> cells at a dose of  $16 \times 10^6$ - $19.5 \times 10^6$  cells/kg (Marktel *et al.* 2017). The median follow-up was 13 months. The procedure was generally well tolerated by all patients, with no product-related adverse events, no evidence of RCL or abnormal clonal proliferation (Marktel *et al.* 2017). Patients showed polyclonal multilineage engraftment, and three of the four treated children became transfusion independent, whereas transfusion requirements decreased in adults, indicating that age of treatment may be an important factor determining the efficacy of the procedure.

Preclinical studies for gene therapy of SCD demonstrated that gene therapy improved sickling. One patient in France was treated with BB305 lentiviral vector transduced HSCs. The patient was followed during two years and became transfusion independent with stable Hb levels of 12 g/dL with therapeutic Hb and HbS accounting for 48% and 46% of the Hb tetramers, respectively (Ribeil *et al.* 2017). In an extended clinical phase I/II trial using this vector, peripheral blood levels of the BB305 vector remained low in all treated SCD subjects, with no evidence of clinical benefit (Kanter *et al.* 2016). In another two gene therapy clinical trials, an anti-sickling  $\gamma/\beta$ -globin transgene (sGbG) (Perumbeti *et al.* 2009) and a  $\beta$ -globin transgene with three anti-sickling point mutations (Lenti-bAS3-FB) (Romero *et al.* 2013) are being assessed currently.

### Metabolic Disorders

Adrenoleukodystrophy (ALD) is an X-linked disease caused by a defect in the ABCD1 (ATP-binding cassette subfamily D) gene which encodes the transporter of the ALD protein (ALDP). ALD was first described in 1992. ALD triggers accumulation of fatty acids that damage the myelin sheaths of neurons, causing motor and cognitive impairment. Allogeneic HSCT is the treatment of choice if a suitable donor is available (Shapiro *et al.* 2000). Lentiviral gene therapy is currently being developed based on promising results showing ALDP expressing human microglia in the brains of NOD/SCID mice after xenotransplantation of lentivirally transduced ALDP expressing human CD34+ HSCs (Benhamida *et al.* 2003). Preliminary results from HSC gene therapy indicated that a limited number of microglia cells may need to be corrected to prevent the demyelinating process (Cartier & Aubourg 2010, Cartier *et al.* 2009). Autologous CD34+ cells genetically corrected using a lentiviral vector encoding the wild-type ABCD1 gene were reinfused into two boys with progressive cerebral demyelination. The boys displayed polyclonal, multilineage engraftment and no evidence of clonal dominance. Cerebral demyelination was arrested at 14 and 16 months, respectively, without further progression (Cartier *et al.* 2012, Cartier *et al.* 2009). In a subsequent phase II/III safety and efficacy clinical study, 17 boys with cerebral X-ALD with early-stage disease and gadolinium enhancement on magnetic resonance imaging (MRI) were infused with autologous CD34+ cells transduced with the elivaldogene tavalentivec (Lenti-D) lentiviral vector. After 29 months of follow-up, all patients had multilineage engraftment of genetically modified cells, with no evidence of preferential integration near known oncogenes or clonal expansion. Although 2 of these 17 patients showed disease progression, the remaining 15 patients had stable expression of the ALD protein (Eichler *et al.* 2017).

Metachromic Leukodystrophy (MLD) is a neurodegenerative lysosomal storage disorder caused by arylsulfatase A (ARSA) deficiency and is autosomal recessively inherited. ARSA deficiency causes accumulation of sulphatide in the CNS, kidney, peripheral nerves, pancreas and liver. The disease affects children and results in premature deaths. Due to their cerebral involvement, both HSCT and development of ERT have been shown to be of limited success (Biffi *et al.* 2011, Boelens, 2006, Boelens *et al.* 2010, Rovelli & Steward 2005). In previous studies, expression of ARSA was detected after transplantation of LV corrected HSCs in a MLD mouse model (Biffi *et al.* 2004, Biffi *et al.* 2006). Recently, a phase I/II clinical trial of MLD was performed in Italy. Three children with ARSA deficiency and mutations associated with early-onset MLD were treated at the presymptomatic stage and received autologous HSCs transduced with a lentivirus carrying the ARSA gene. All three patients showed high-level polyclonal, multilineage engraftment of the transduced HSCs and

high ARSA activity was detected in the hematopoietic lineages and in the cerebrospinal fluid resulting in arrested progression of neurodegenerative symptoms (Biffi *et al.* 2013). No evidence of clonal expansion was detected, indicating that gene therapy may be a good alternative to HSCT, especially since the effects of ERT for storage diseases with cerebral brain involvement are limited.

For diseases such as Gaucher, Fabry and Pompe disease, ERT is available and effective. However, the development of gene therapy may still serve a purpose because of the impact on the patient (requiring recurrent visits to the hospital for ERT infusion), the cost-effectiveness of the procedure (ERT is relatively expensive in comparison to a single gene therapy treatment) and because gene therapy of autologous HSCs is in intention a single, curative treatment and minimally invasive, increasing the quality of life of patients.

For other metabolic diseases for which allogeneic HSCT is possible, in the absence of a matched donor, gene therapy offers the advantages of decreasing morbidity related with allogeneic HSCT such as myeloablative conditioning and GvHD. Ongoing developments have made the used lentiviral vectors increasingly safe and promising results are obtained in clinical trials in most of the inherited monogenic diseases. An overview of the results of gene therapy trials for diseases discussed above is given in Table 2.

### New developments for improved lentiviral vector biosafety and efficacy

Gamma retroviral vectors have been shown to preferentially integrate near transcriptional start sites and regulatory gene regions of specific proto-oncogenes, such as *LMO-2*, *CCND2*, *MDS1/EV11*, *PRDM16*, *SETBP1*, *MECOM* (Howe *et al.* 2008) and many of the initial clinical trials for X-linked SCID, CGD and WAS have shown the risks related to the use of  $\gamma$ RV (Ghosh & Gaspar 2017, Hacein-Bey-Abina *et al.* 2008, Howe *et al.* 2008, Ott *et al.* 2006). To reduce the risks of insertional mutagenesis, third generation self-inactivating retroviral and lentiviral vectors were developed (Maetzig *et al.* 2011), which have been shown to have a significantly improved safety profile (Bordignon *et al.* 1995, Cartier *et al.* 2012, Cartier *et al.* 2009, Cavazzana 2016, Hacein-Bey Abina *et al.* 2015, Kohn *et al.* 1998, Morris *et al.* 2017, Thompson *et al.* 2016).

Although the SIN-LV vectors, which display a preferred integration in transcribed genes, are considered less genotoxic than the SIN- $\gamma$ RV vectors, the risks of insertional transformation of HSCs by both SIN-LV and SIN- $\gamma$ RV vectors remains present and appears to be dictated largely by the type of internal promoter used and the transgene itself (Modlich *et al.* 2009). Physiological (internal) promoters are generally weaker insertional mutagens in comparison to retroviral promoters (Zychlinski *et al.* 2008) and using lineage or tissue-specific promoters can therefore potentially increase biosafety (Pauwels *et al.* 2009). Furthermore,



the use of tissue-specific promoters or inducible promoters over constitutively active promoters may result in a more physiological expression pattern of the transgene, which may be preferred to avoid cellular toxicity related to overexpression of transgenic proteins (Arnold & Heimall 2017), as well as to avoid an immunological response against the transgenic protein. *In vitro* quantitative assays, such as immortalization tests, demonstrated that the risk of insertional activation of proto-oncogenes is directly related to the strength of the enhancer sequences contained in the vector (Schambach *et al.* 2013). One way to overcome this problem is to introduce insulators, such as cHS4, which can establish boundaries between regulatory sequences, into the viral genome (Gaszner & Felsenfeld 2006,

Puthenveetil *et al.* 2004), both preventing activation of nearby proto-oncogenes and at the same time protecting the transgene from silencing by spreading heterochromatin. The design of novel pseudotypes may further increase cell-specific targeting during transduction of mixed populations of unstimulated hematopoietic stem and progenitor cells, which will improve transduction efficiency (Frecha *et al.* 2008). In addition, current developments with regards to direct targeting of viral integration into “safe sites” of the genome (gene addition) or targeted integration into the site of the mutated gene (gene replacement) using zinc finger nucleases (Porteus & Carroll 2005) or CRISPR-Cas9 nucleases will greatly improve physiological regulation of the transgenic gene and biosafety.

**Table 2.** Results from clinical trials of hematopoietic stem cell gene therapy for inherited monogenic diseases.

Disease	Phenotype	Affected gene	Vector	Current Rx	Side effects	Clinical benefit	References
<b>X-SCID</b>	Absence of T and NK cells and mature B cells	IL2R $\gamma$	$\gamma$ RV	HSCTx	Insertional mutagenesis	Selective growth advantage of transduced cells Immunological reconstitution	(Cavazzana-Calvo <i>et al.</i> 2000, Hacein-Bey-Abina <i>et al.</i> 2008, Hacein-Bey-Abina <i>et al.</i> 2002, Howe <i>et al.</i> 2008)
<b>ADA-SCID</b>	Immunodeficiency neurological abnormalities	ADA	$\gamma$ RV	ERT HSCTx	-	Selective growth advantage of transduced cells, immunological reconstitution, clinical improvement, discontinuation of ERT treatment	(Aiuti <i>et al.</i> 2007, Aiuti & Roncarolo 2009, Cappelli & Aiuti 2010, Ferrua <i>et al.</i> 2010b)
<b>X-CGD</b>	Non-functional phagocytes	Gp91phox	SIN-LV	HSCTx	Insertional mutagenesis	No selective growth advantage of transduced cells Cellular toxicity, loss of corrected HSCs	(Keller <i>et al.</i> 2018, Stein <i>et al.</i> 2010)
<b>WAS</b>	Microthrombocytopenia, autoimmunity	WASP	$\gamma$ RV SIN-LV	HSCTx	Insertional mutagenesis -	- Multilineage engraftment, clinical improvement, no clonal expansion	(Ghosh & Gaspar 2017) (Hacein-Bey Abina <i>et al.</i> 2015, Morris <i>et al.</i> 2017)
<b><math>\beta</math>-Thal</b>	Hemolytic Anemia	$\beta$ -globin	SIN-LV	HSCTx	-	Polyclonal integration, no clonal dominance, multilineage engraftment, transfusion independence	(Cavazzana-Calvo <i>et al.</i> 2010, Cavazzana 2016, Thompson <i>et al.</i> 2016)
<b>SCD</b>	Hemolytic anemia	$\beta$ -globin	SIN-LV	HSCTx	-	Minimal clinical benefit	(Marktel <i>et al.</i> 2017)
<b>X-ALD</b>	Cerebral demyelination	ABCD1	SIN-LV	-	-	Arrested progression of demyelination, polyclonal, multilineage engraftment and no clonal dominance	(Cartier <i>et al.</i> 2012, Cartier <i>et al.</i> 2009, Eichler <i>et al.</i> 2017, Kohn <i>et al.</i> 1998)
<b>MLD</b>	Cerebral demyelination	ARSA	SIN-LV	HSCTx	-	High-level polyclonal, multilineage engraftment, arrested progression of neurodegenerative symptoms, no clonal expansion	(Biffi <i>et al.</i> 2013, Bordignon <i>et al.</i> 1995)

### Current status of gene therapy research in Turkey

Very few research groups in Turkey are currently working on the development of gene therapeutical approaches for inherited diseases. According to the website of the Scientific and Technological Research Council of Turkey (TÜBİTAK, <https://www.tubitak.gov.tr>) genetic modification related R&D activities were concentrated in projects in the Genetic Engineering and Biotechnologies Institute of MAM (Marmara Research Center). These projects however, were more focused on the development of different transgenic mouse models, development of vaccines or development of non-viral vectors. Search queries for research projects using “lentiviral” and “vector” as search items using the TÜBİTAK database for supported projects ([www.cabim.ulakbilim.gov.tr/tr-dizin/tubitak-destekli-projeler-veritabanı](http://www.cabim.ulakbilim.gov.tr/tr-dizin/tubitak-destekli-projeler-veritabanı)) resulted in a total of 60 hits, of which only 1 focused on gene therapy. However, since this database has not been updated since 2013, these numbers are most likely not representative for the actual numbers of currently ongoing gene therapy related projects.

### Why is the development of gene therapy important for Turkey?

Consanguinous marriages increase the risk of autosomally inherited diseases. The highest rates of consanguinous marriages occur in North and sub-Saharan Africa, the Middle East, and West, Central and South Asia. According to the Turkish statistical institute statistic (TÜİK), the rate of consanguinous marriage rates in Turkey is approximately 21%. Of these, 35% are located in rural areas and 20-25% are located in urban areas. Within Turkey, the rates of consanguinous marriages are highest in Southeastern Anatolia (40.4%) and lowest in Western Marmara (4.8%). Overall in Turkey, one in five marriages is between closely related family members, of which 70% between first cousins. Marriages between first and second cousins increases the risk of post-natal infant mortality significantly, with 45.9/1000 infant deaths in unrelated marriages, versus 72.1 infant deaths in consanguineous marriages (Sağlık Bakanlığı, 2002). This high level of consanguinity in Turkey results in a relatively high prevalence of PIDs and metabolic diseases in comparison to Europe and the USA and annually 4000 patients with possible PIDs are referred to the 10 pediatric immunology centers in Turkey. In addition, according to an official publication by the Ministry of Health in 1996, the number of patients with diseases of blood or blood-forming organs, excluding anemias, reached 9597 within the years 1964-1994. Thus, the social economic burden of the not so rare diseases in Turkey is quite substantial.

For some of the inherited monogenic diseases allogeneic HSCT can be curative, and where outside of Turkey the search for an available, suitable donor may be difficult; in Turkey often matching donors can be found within families (Balci *et al.* 2011). However, even if available, both HSCT and ERT-related morbidity and frequent hospital visits, make the development of a single,

curative treatment a tempting alternative. In addition, the costs related to HSCT, including conditioning and follow-up, and life-long treatment with ERT are considerable: In the USA, costs related to life-long ERT treatment for Fabry disease (Fabrazyme) are estimated to be \$200,000/year per patient; Hurler Syndrome (Aldurazyme) \$200,000/year per patient; Gaucher type 3 (Cerezyme) \$200,000/year per patient; Pompe disease (Myozyme) \$300,000/year per patient; Hunter Syndrome (Elaprase) \$375,000/year per patient. Whereas the national health insurance systems in West Europe fully reimburse costs related to HSCT or ERT, other countries may not or not completely cover costs related to orphan drugs (Shah 2006), making the development of a single, curative treatment, such as gene therapy for these countries a high priority.

### **Recommendations for Turkey**

The rationale of gene therapy is to repair, inactivate or to replace dysfunctional genes that cause disease with the aim of establishing or acquiring normal function. Hematopoietic stem cell gene therapy offers a possible curative treatment option for a range of patients with inherited monogenic diseases, who currently have no alternative treatment option, such as HSCT or ERT. Many gene therapy clinical trials have been performed mostly in Europe and the USA during the last twenty years for a wide range of immune deficiencies and metabolic diseases, of which only a fraction is discussed above. Although several Turkish patients have been treated with gene therapy for ADA-SCID, CGD and WAS, and patients enrolled in gene therapy clinical trials include a disproportionate number of children from Turkish origin, development of gene therapy or participation in gene therapy clinical trials for inherited monogenic diseases in Turkey is not currently actively pursued.

Gene therapy not only offers a curative treatment option, but also, as observed with the latest SIN-LV constructs, provides in general a good quality of life, low treatment-related morbidity, a decreased duration and frequency of hospitalization in addition to an improved clinical condition. However, the efficacy of the treatment is not only related to the number of cells infused, the vector copy number per cell or the level of transgene expression, but even more to the general condition of the patient upon start of the treatment. When gene therapy is done in eligible patients as soon as possible after diagnosis and preferably before irreversible symptoms occur, these patients have the best chances of good recovery and clinical improvement (Aiuti & Roncarolo 2009, Bordignon *et al.* 1995, Marktel *et al.* 2017, Thrasher *et al.* 2005).

Although current lentiviral vectors still harbor the intrinsic risk of insertional mutagenesis, recent clinical trials have shown that the actual occurrence of myelodysplastic syndromes and leukemogenesis is very low and that the benefits of the treatment outweigh the risks. Obviously, the long-term risks of the procedures cannot be fully appreciated, since gene therapy is just

coming of age, and assessment of these risks will require carefully planned, long-term follow-up of all treated patients. However, future improvements resulting in tissue-specific expression of genes, replacement of genes rather than addition, and increased biosafety will make hematopoietic stem cell gene therapy a powerful new tool to cure previously incurable patients. Therefore, the establishment of specialized centers in Turkey for gene therapy research, for the development of clinical gene therapy or participation in phase I/II gene therapy studies and for the production and quality control of gene therapy products should be stimulated.

In conclusion, lentiviral treatment of rare, inherited diseases is being rapidly developed and optimized and

currently tested in multicenter preclinical trials. Although past experiences with retroviral and lentiviral gene therapy have shown that results should be assessed carefully, and long-term follow-up remains required, current data are very promising. Therefore, we recommend development of a specialized research center infrastructure that would allow participation of Turkey not only by providing patients or patient samples, but also by taking part at the research level and in the preclinical trials.

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