# Lentiviral Hematopoietic Stem Cell Gene Therapy for MNGIE

Rana Yadak

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Lentivirale hematopoietische stamcelgentherapie voor MNGIE

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Cover design: Rana Yadak, the traditional Palestinian embroidery pattern "wide open eye [✻]" assembled as nucleobases in the DNA ladder, with a background of the human TYMP gene sequence.

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# Lentiviral Hematopoietic Stem Cell Gene Therapy for MNGIE

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إهداء للوالد و الوالدة

*For my father and mother.*

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# General introduction

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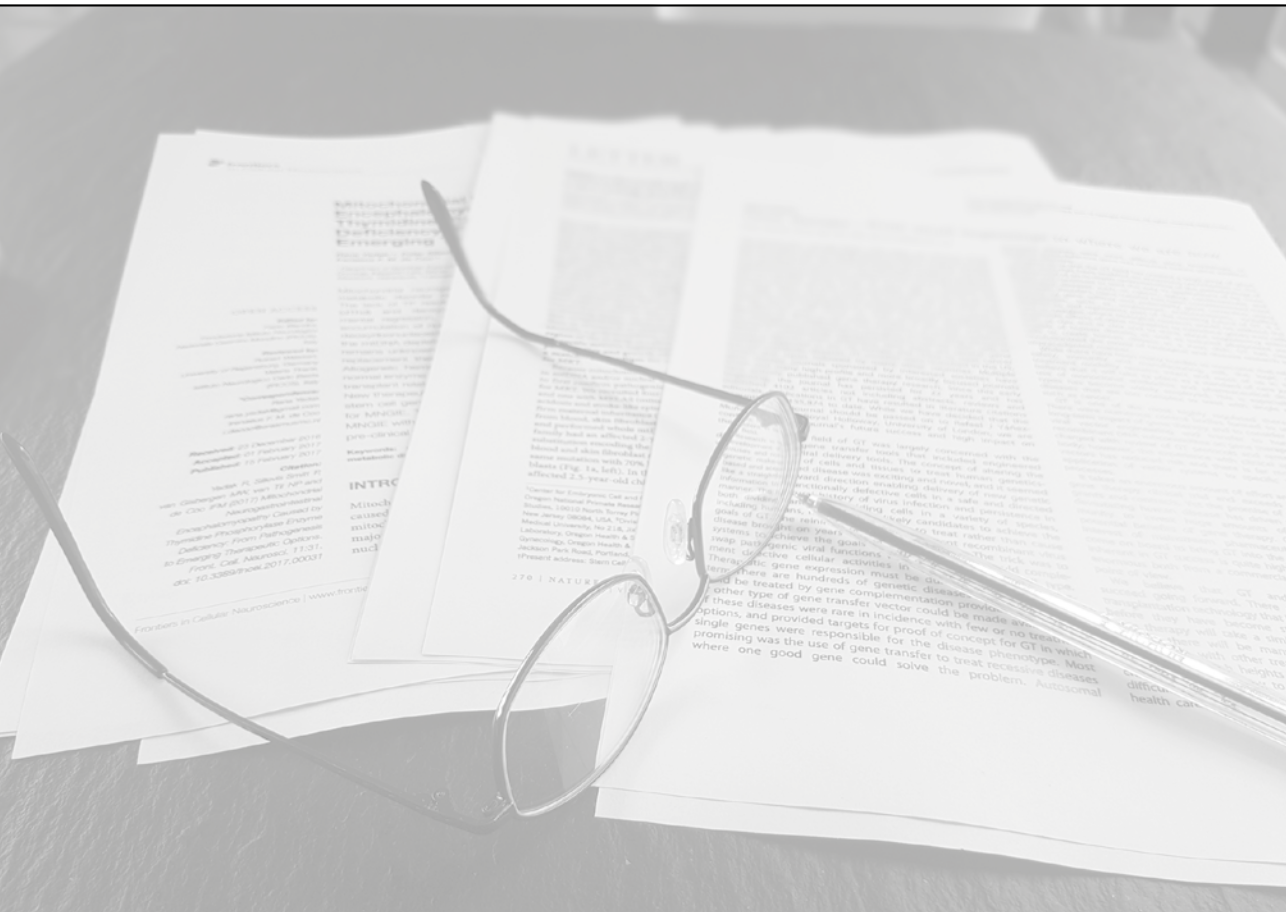

## | HEMATOPOIETIC STEM CELL GENE THERAPY (HSCGT)

Hematopoietic stem cells (HSCs) are multipotent stem cells with dual functional attributes of self-renewal to retain the stem cell pool and to differentiate into cells of the immune system and blood through hematopoiesis.[1,2] The bone marrow (BM) niche is the microenvironment where quiescent HSCs reside, hematopoiesis occurs, and from which HSCs mobilize into the periphery and home to and engraft after BM transplantation. Primary stromal cells are involved in these processes, in which adhesion molecules, chemokines, and cytokines play a major role (reviewed in Lapidot T *et al.* 2002, 2005).[3,4] This forms the basis of hematopoietic stem cell transplantation (HSCT). HSCT is a common clinical procedure to reconstitute the hematopoietic/immune system after conditioning by radiation or chemotherapeutic regimens. Allogeneic HSCT involves the transplantation of donor matched-BM, mobilized peripheral blood, or umbilical cord blood (UCB)-derived HSCs, and is the therapy of choice for a variety of (non-) malignant hematological and non-hematological conditions.[5] Graft versus host disease, graft failure, and toxicity of the preparative regimens are common complications related to allogeneic HSCT. Autologous hematopoietic stem cell gene therapy (HSCGT), the *ex vivo* transfer of a functional copy of a gene (therapeutic gene) into the patient's own hematopoietic stem/progenitor cells (HSPCs), is another application of HSCT.

The family of retroviruses contains two members that form the basis of viral vectors used in clinical trials: i.e. gammaretrovirus (γ-RV) and lentivirus (LV), which have been successfully used for HSCGT due to their ability to permanently integrate their DNA into the host HSPCs' genomic DNA. LV vectors offer several advantages over γ-RV vectors. First, LVs possess a central polypurine tract (cPPT) element which significantly improves nuclear import of the pre-integration complex,[6] therefore LV vectors can transduce non-dividing quiescent HSCs. The capacity to transduce non-dividing HSCs significantly reduces the need for excessive *ex vivo* HSPCs cytokine stimulation to proliferate compared with γ-RV vectors, which is detrimental for the long term repopulation of gene modified HSCs *in vivo*.[7] Second, insertional oncogenesis has been observed in clinical trials for X-linked SCID, Wiskott-Aldrich syndrome, and chronic granulomatous disease using γ-RV.[8-12] In contrast, animal studies showed that the LV integration profiles are relatively safe compared to γ-RV as demonstrated with adrenoleukodystrophy.[13] Due to these advantages, HSCGT has recently been applied successfully in several hematological, immunological and metabolic disorders.[14] The rationale for using HSCGT to target metabolic diseases relies on the capacity of the gene modified HSPCs and their progeny to act as a factory of the therapeutic proteins. The protein's substrate can be converted in the gene modified cells or the protein excreted and delivered through cross-correction. HSCs' self-renewal capacity and the highly proliferative progenitors mediate the long lasting effects of HSCGT. The rationale for and the clinical procedure of autologous LV-based HSCGT are presented in **Figure 2A, Chapter 2**.

## MITOCHONDRIAL NEUROGASTROINTESTINAL ENCEPHALOMYOPATHY (MNGIE)

MNGIE is a rare metabolic disease caused by mutations in the *TYMP* gene, which results in defective thymidine phosphorylase (TP) enzyme activity and subsequent increase in its nucleoside substrates, deoxyribonucleosides thymidine (dThd) and deoxyuridine (dUrd). This consequently results in altered mitochondrial DNA (mtDNA). Due to the limited therapeutic efficacy of the available treatments and the severe toxicity related to allogeneic HSCT, gene therapy emerged as an attractive alternative for treatment of MNGIE. The experiments performed in this thesis were designed to evaluate the efficiency and safety of LV based HSCGT for MNGIE. Genetic defects, clinical manifestations, diagnosis, pathogenesis, current treatments, pre-clinical studies, and future directions in MNGIE research are discussed in **Chapter 2**.

# | SCOPE AND OUTLINE OF THIS THESIS

To cure rare inherited diseases, HSCGT has emerged as a promising platform for numerous hematological, immunological, and metabolic disorders[1] (reviewed in Naldini, 2015).[14] The currently investigated treatment options for MNGIE often only provide temporary restoration of biochemical homeostasis that lacks long-term improvement of clinical phenotypes, such as infusion of platelets and peritoneal dialysis. Allogeneic HSCT could be a potential curative treatment, but is associated with substantial morbidity and mortality (discussed in **Chapter 2**). A recent approach to performing liver transplantation has obvious drawbacks, such as life-long immune suppression and graft failure. Alternative therapies that provide life-long effective biochemical and phenotypic correction with acceptable adverse effects are an urgent medical need. The studies performed in this thesis were designed to evaluate the potential application of LV based HSCGT as a safe and efficient treatment for MNGIE.

## *(i)* Optimization of culture conditions for highly efficient LV transduction of HSPCs without compromising long term repopulation efficiency and gene marking *in vivo*.

Most HSPC transduction protocols use multiple rounds of LV vector incubations with high vector doses, yielding many integrated LV copies per genome, and as a consequence, a relatively high genotoxicity risk. Also, high concentrations of multiple cytokines are used to induce HSPC proliferation and enhance transduction efficiency. Therefore, the development of highly efficient LV transduction protocols for HSPCs are necessary. Optimizing parameters such as the duration of the transduction, reducing the number of integrations per cell, and restriction of growth factors, would increase the number of patients that could be treated per LV vector stock, thereby reducing the costs substantially. Furthermore, this could potentially also improve long-term therapeutic outcomes. To address these points, the following experiments were designed as described in **Chapter 3**. First, lineage-depleted (Lin-) mouse BM cells, rhesus BM CD34+ cells, and human peripheral blood and BM CD34+ progenitor cells, were transduced overnight at an increasing cell density with a proportional increase of transducing units in order to increase their physical contact. Second, lin- cells and human umbilical cord blood CD34+ cells were transduced overnight under varying concentrations of selected growth factors either singularly or in combinations. The repopulation capacity and stable gene marking *in vivo* was assessed by the transplantation of lin- cells into sublethally irradiated wild type recipient mice.

## *(ii)* Efficiency assessment of LV based HSCGT for MNGIE

Initial attempts demonstrated correction of the biochemical phenotype in a mouse

[1] https://clinicaltrials.gov

model of MNGIE following LV based HSCGT,[15] encouraging its further pre-clinical development as a treatment option for MNGIE. We aimed at addressing the efficiency in depth, particularly on neurological and intestinal phenotypes which are otherwise unresponsive to allogeneic HSCT. We tested third generation self-inactivating LV vectors with a backbone similar to those applied in clinical trials with a modified Woodchuck post-transcriptional regulatory element, and the cellular human phosphoglycerate kinase (hPGK) promoter to drive the human (*TYMP*) or codon optimized (*TYMPco)* cDNA sequences (**Figure 1A, Chapter 4**). The efficacy of HSCGT was tested in *Tymp*$^{-/-}$*Upp1*$^{-/-}$ for up to 8 - 11 months. Dose scaling of LV vectors and transplanted gene modified cells was performed to establish the minimum required VCN/cell sufficient for biochemical correction. The evaluation of therapeutic outcomes (**Chapter 4**) involved assessments of ***(a)*** molecular chimerism and integrated LV vector copies; ***(b)*** biochemical phenotype in urine, blood, brain, intestine, liver, and muscle tissues; ***(c)*** neurological phenotypes by performing memory and motor function assessments, brain MRI and immunohistochemistry; and in **Chapter 5** ***(d)*** we assessed the pathology of the small intestine of mice receiving HSCGT as well as MNGIE patients treated with allogeneic HSCT.

### *(iii)* Safety assessment of LV based HSCGT for MNGIE

To address any potential side effects related to TP overexpression or LV- related genotoxicity, we performed experiments as described in **Chapters 4 and 6**: ***(a)*** *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice transplanted with gene modified HSPCs bearing LV vectors with a strong promoter/enhancer derived from spleen focus forming virus (LV-SF) were monitored long term. ***(b)*** Long term follow up was performed on *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice recipients of HSPCs transduced with a therapeutic vector containing the PGK promoter. Both under ***(a)*** and ***(b)*** the discomfort was monitored and hematological analysis was performed to exclude hematological abnormalities. ***(c)*** Secondary transplantations were performed to assess tumor incidence up to 11 months. ***(d)*** LV integration sites were retrieved by LAM-PCR on genomic DNA of lin- cells and in BM cells after transplantation.

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# 2

# Mitochondrial Neurogastrointestinal Encephalomyopathy Caused by Thymidine Phosphorylase Enzyme Deficiency: From Pathogenesis to Emerging Therapeutic Options

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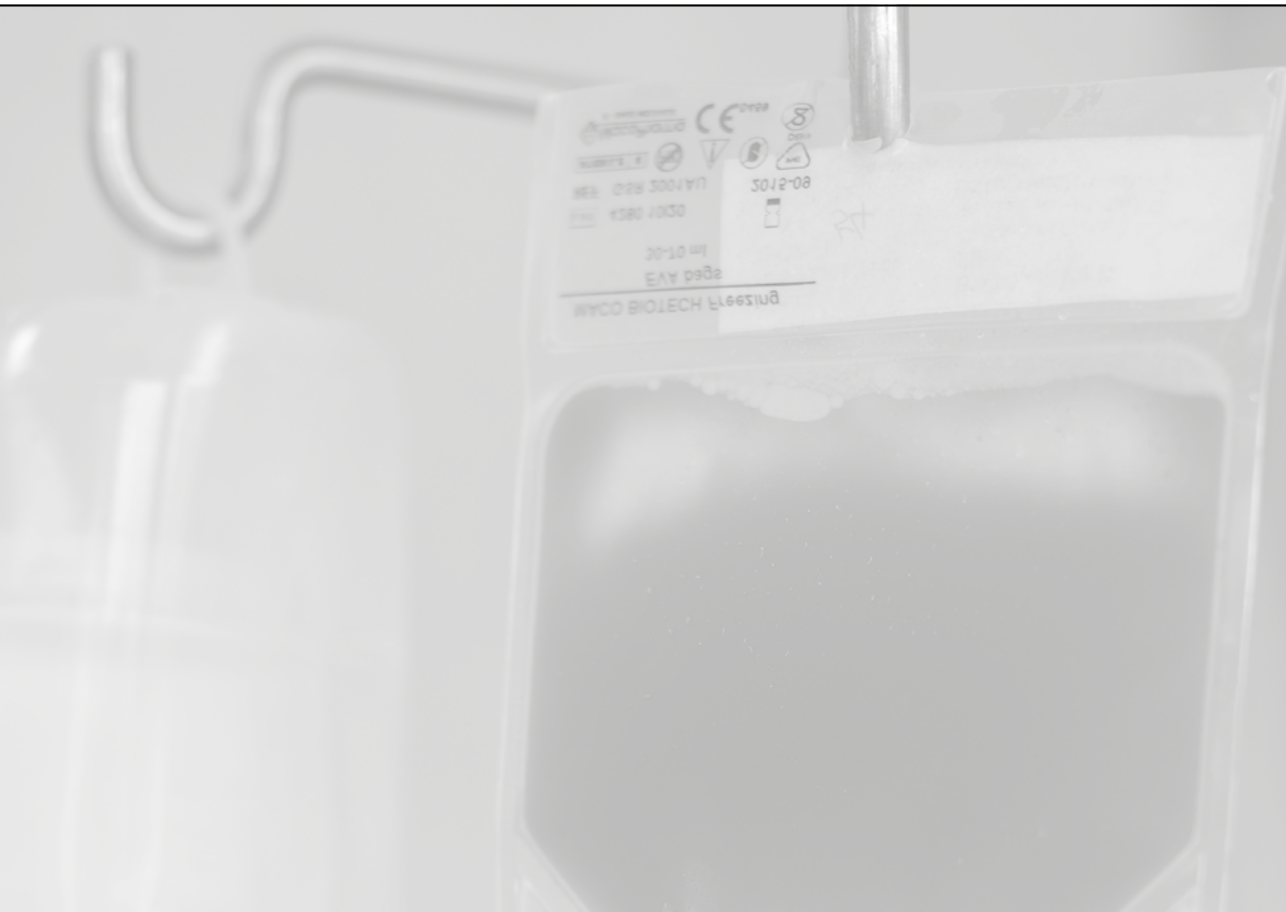

## ABSTRACT

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is a progressive metabolic disorder caused by thymidine phosphorylase (TP) enzyme deficiency. The lack of TP results in systemic accumulation of deoxyribonucleosides thymidine (dThd) and deoxyuridine (dUrd). In these patients, clinical features include mental regression, ophthalmoplegia and fatal gastrointestinal complications. The accumulation of nucleosides also causes imbalances in mitochondrial DNA (mtDNA) deoxyribonucleoside triphosphates (dNTPs), which may play a direct or indirect role in the mtDNA depletion/deletion abnormalities, although the exact underlying mechanism remains unknown. The available therapeutic approaches include dialysis and enzyme replacement therapy, both can only transiently reverse the biochemical imbalance. Allogeneic hematopoietic stem cell transplantation (AHSCT) is shown to be able to restore normal enzyme activity and improve clinical manifestations in MNGIE patients. However, transplant related complications and disease progression result in a high mortality rate. New therapeutic approaches, such as adeno-associated viral (AAV) vector and hematopoietic stem cell gene therapy have been tested in *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice, a murine model for MNGIE.

This review provides background information on disease manifestations of MNGIE with a focus on current management and treatment options. It also outlines the pre-clinical approaches towards future treatment of the disease.


Key words: mitochondrial neurogastrointestinal encephalomyopathy; MNGIE; thymidine phosphorylase; metabolic disease; HSCT; HSCGT; lentiviral vector

# | INTRODUCTION

Mitochondrial diseases represent a genetically and clinically heterogeneous group of disorders caused by mutations in mitochondrial DNA (mtDNA), that affect synthesis and function of mitochondrial proteins, such as tRNA (in MELAS disease) and ND1, 4, 6 (responsible for the majority of cases in LHON disease) (DiMauro, 2004). Another group is caused by mutations in nuclear DNA (nDNA) that lead to defects in nuclear encoded mitochondrial proteins. Part of these proteins exert their effect on mtDNA maintenance, thus known as nuclear-mitochondrial communication disorders. A subtype of the latter is mitochondrial DNA depletion syndrome (MDS); a group of mainly autosomal recessive disorders caused by defects in nuclear genes involved in mtDNA replication (e.g. *POLG* and *PEO1* causing hepatocerebal MDS), or genes crucial for maintenance of mtDNA including *TK2* (responsible for myopathic MDS*), RRM2B* (encephalomypathic MDS) and thymidine phosphorylase (*TYMP*) gene mutations associated with MNGIE (El-Hattab and Scaglia, 2013). MNGIE, initially described in 1976 by Okamura (Okamura et al., 1976) is a fatal rare inherited metabolic disorder without genetic or ethnic predisposition (Gamez et al., 2005). The estimated rate of occurrence is 1-9:1,000,000[1], and as of 2011 fewer than 200 cases have been described in the medical literature (Halter et al., 2011). Due to its variable clinical presentations, MNGIE can be easily overlooked or misdiagnosed as Crohn's disease, psychiatric disorder, anorexia nervosa or myasthenia gravis (Rickards et al., 1994;Teitelbaum et al., 2002; Marti et al., 2004).

# | GENETIC DEFECTS, CLINICAL MANIFESTATIONS AND DIAGNOSIS

## Genetic defects

MNGIE is an autosomal recessive inherited disease that is caused by mutations in the nuclear gene *TYMP* (previously known as *ECGF1*). *TYMP* codes for the TP enzyme (EC 2.4.2.4.) and is located on chromosome 22q13.33 (Stenman et al., 1992).TP is a cytoplasmic enzyme expressed in most human tissues, including gastrointestinal tract, central and peripheral nervous system, spleen, liver, bladder, leukocytes and in platelets which account for most of the TP activity in human blood (Fukami and Salganicoff, 1973;Shaw et al., 1988). In contrast, TP is present at low levels in muscles and is lacking in kidney, aorta and fat tissues (Fox et al., 1995;Valentino et al., 2007). MNGIE is caused by a variety of pathogenic homozygous or compound heterozygous mutations in the exons or flanking regions of the *TYMP* gene. Various mutations are reported to date (Stenson et al., 2014) including deletions, single nucleotide insertions (Nishino et al., 1999), splice site (Kocaefe et al., 2003;Szigeti et al., 2004b) and frameshift mutations (Blazquez et al., 2005)

[1] http://www.orpha.net/consor/cgi-bin/index.php

and a homozygous duplication mutation in exon 8 of the *TYMP* gene (Gamez et al., 2005). The majority of these mutations are loss of function mutations. Heterozygous mutation carriers are asymptomatic with approximately 35% residual TP activity, although the plasma nucleoside levels are similar to healthy controls (Marti et al., 2004).

In addition, non-pathogenic polymorphisms have been described in the *TYMP* gene. The A465T polymorphism (c.1393G>A) was reported both in subjects with MNGIE like features and control subjects (Vissing et al., 2002;Martin et al., 2004). In some MNGIE cases there is no or mild clinical involvement of gastrointestinal tract or skeletal muscle, despite the presence of mutations in the *TYMP* gene leading to marked reduction in TP activity, probably indicating that environmental factors contribute to the severity of the clinical symptoms (Martin et al., 2004;Szigeti et al., 2004b). Apart from late-onset forms of the disease (Marti et al., 2005;Massa et al., 2009;Etienne et al., 2012), most patients display typical MNGIE features before the age of 20 years (Nishino et al., 2000;Teitelbaum et al., 2002).

## Clinical manifestations

Gastrointestinal and ocular involvement are usually the first complications in this disease, although neuropathy and hearing loss have been reported as primary symptoms in some cases (Garone et al., 2011). Clinical symptoms are summarized in **Table 1**.

## Diagnosis

Detailed patient history, thorough clinical examination, particular findings on magnetic resonance imaging (MRI) of the brain (**Figure 1**), genomic DNA screening for mutations in *TYMP* gene and biochemical analysis all contribute to the diagnosis of MNGIE. Biochemical diagnosis of MNGIE includes at least one of the following parameters (Marti et al., 2004): (1) Increased blood plasma levels of dThd and dUrd (>3μmol/L and >5μmol/L respectively). (2) Severely reduced TP enzyme activity in buffy coat leukocytes (<8% of healthy controls; healthy control mean TP activity equivalent to 634 nmol thymine formed /hr/mg protein). Biochemical analysis reduces the risk of missing the diagnosis in case of non-identified mutation sites (Nishino et al., 2000) or in case of unclassified variants (UV). Additionally, biochemical diagnosis contributes to the confirmation or exclusion of the the role of a UV as a cause for MNGIE. Similarly, biochemical assessment is preferred over clinical diagnosis since some of the classical symptoms of MNGIE can be absent. Other frequently observed findings in MNGIE patients include metabolic abnormalities such as lactic acidosis, deficiency of mitochondrial respiratory chain enzymes, mainly complex I and IV (Hirano et al., 1994) (Debouverie et al., 1997), urinary Thd and dUrd accumulation (Fairbanks et al., 2002;Spinazzola et al., 2002;la Marca et al., 2006) and elevated protein levels in CSF (Bedlack et al., 2004). Infrequently, skeletal muscle biopsies may reveal ragged red fibers, and mtDNA analysis may reveal acquired deletions, depletions or point mutations (Teitelbaum et al., 2002;Nishigaki et al., 2003).

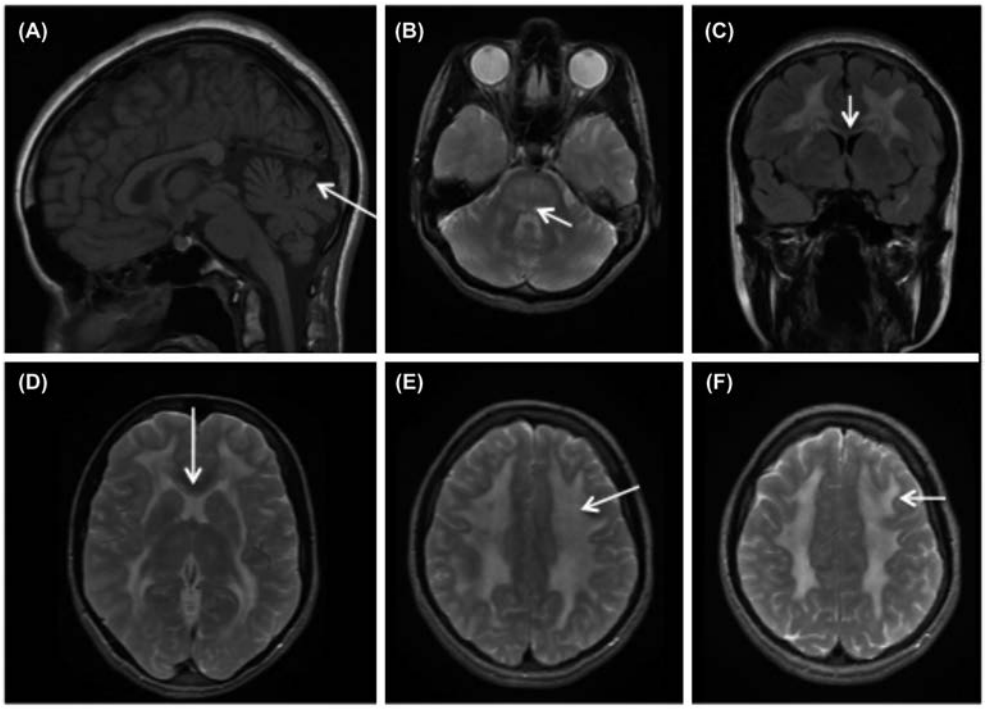


**Figure 1. Brain MRI findings in MNGIE.** MRI of MNGIE patient at age 16 with " typical" MNGIE phenotype . **(A)** T1 weighted sagital image shows cerebellar vermis atrophy (arrow) and normal gyral pattern. **(B)** Axial T2 with hyperintensities in the dorsal pons and mesencephalon (arrow). (**C** coronal flair image, **D** axial T2) show extensive signal abnormalities in the cerebral white matter. The external capsule is involved as is the inner blade of the corpus callosum (arrow **C**, **D**). **(E**, **F)** Extensive white matter involvement with sparing of the U-fibers (arrow).

## | PATHOGENESIS

The TP enzyme converts mitochondrial dThd and dUrd to the nucleotide bases thymine and uridine respectively and 2-deoxy ribose 1-phosphate (Friedkin and Roberts, 1954). This occurs in *de novo* synthesis or via the salvage pathway. dThd and dUrd are homogeneously present in cellular and plasma compartments and they translocate between compartments through nucleoside transporters (NTs). In humans two unrelated protein families have been described (Young et al., 2013), concentrative nucleoside transporters (CNTs), an active transport system, and equilibrative nucleosides transporters (ENTs) responsible for passive facilitated diffusion.

The bidirectional ENTs, mainly ENT1, are ubiquitously present on almost all cell types and mediate the uptake and efflux of nucleosides **(Figure 2B)**. Therefore they are important for cells that rely on the salvage pathway for supply of nucleosides, including bone marrow cells, erythrocytes and leukocytes, brain and muscles (Young et al., 2008). Although TP is not expressed in all tissues, the TP expressed in circulating

**Table 1.** Common and rare clinical symptoms in MNGIE patients.

| Complication | Symptoms | Pathophysiology |
|---|---|---|
| Gastrointestinal | Appetite loss, satiety<br>Weight loss<br>Digestive features: Chronic diarrhea, abdominal pain, cramps, nausea, colonic distension, dysphagia | Myogenic (visceral smooth muscle):<br>atrophy in the muscularis propria of the stomach and small intestines.<br>Neurogenic (enteric nervous system): loss of the interstitial cells of Cajal.<br>Mixed myo-neurogenic causes |
| Ocular | External ophthalmoplegia, ptosis, retinal pigmentary changes, glaucoma, optic nerve atrophy | |
| Auditory | Deafness | Dysfunction of cranial nerve and auditory cortex.<br>Atrophy of the stria vascularis in the cochlea |
| CNS | Mental changes, subcortical loss of cognitive functions, memory impairment | leukencephalopathy |
| PNPs | Numbness and paraesthesia | Demyelinating sensorimotor type: reduced sensory motor conduction, loss of myelin sheaths in lumbar and brachial plexus. |
| Skeletal muscle | Proximal myopathy | mtDNA molecular alterations and abnormal respiratory chain enzymes in skeletal muscles |
| Others | Endocarditis<br>Spontaneous abdominal esophageal perforation<br>Short stature<br>Cardiomyopathy<br>Psoriasis | |

CIPO, Chronic intestinal pseudo obstruction; CPEO, Chronic progressive external ophthalmoplegia; CNS, central nervous system; PNPs, polyneuropathies

| Remarks | Study |
|---|---|
| A major cause of death and survival is generally related to the severity of these symptoms.<br>Can lead to severe denutrition, anaemia and eventually the necessity for nutritional supportive treatments.<br>CIPO in the early disease course is under recognized. | (Granero Castro et al., 2010),(Garone et al., 2011),(Giordano et al., 2008),(Zimmer et al., 2009) ,(Perez-Atayde et al., 1998) ,(Blondon et al., 2005),(Chapman et al., 2014). |
| CPEO phenotype is often present. Recovered upon HSCT transplantation compared to untreated patient. | (Threlkeld et al., 1992),(Barboni et al., 2004),(Vinciguerra et al., 2015) |
| Hearing loss is common among patients (in 61% of patients).<br>Satisfactory results were obtained soon following cochlear implantation in MNGIE patients. | (Hirano et al., 1994), (Yasumura et al., 2003), (Li et al., 2011), (Mattman et al., 2011) |
| MNGIE is an example of an adult mitochondrial disorder in which leukodystrophy is observed.<br>Patients presenting the characteristic multisystem symptoms of MNGIE have a unique pattern on brain MRI indicative of vasogenic oedema and glial cell dysfunction.<br>To date, it is debatable whether or not the extent of these brain MRI signal alterations, correlates with age, clinical severity, CNS involvement or the biochemical and genetic profiles of MNGIE patients. | (Millar et al., 2004),(Barragan-Campos et al., 2005),(Scaglia et al., 2005) ,(Schupbach et al., 2007),(Schiffmann and van der Knaap, 2009) ,(Scarpelli et al., 2013),(Carod-Artal et al., 2007;Salsano et al., 2013) |
| Neuropathy usually is not among the first symptoms of the disease.<br>Some MNGIE cases are misdiagnosed with chronic inflammatory demyelinating polyneuropathy. | (Simon et al., 1990),(Hirano et al., 1994),(Bedlack et al., 2004),(Menezes and Ouvrier, 2012),(Pupe et al., 2012) |
| Two cases with classical clinical presentation of MNGIE, were reported without skeletal muscle involvement.<br>Both cases showed identical homozygous splice-acceptor site mutation in *TYMP* gene (c.215-1G>C), which may suggest a genotype-phenotype correlation. | (Papadimitriou et al., 1998),(Hirano et al., 2004), (Szigeti et al., 2004b;Cardaioli et al., 2010; Bax et al., 2013) |
| Rare complications<br>Short stature as seen in many mitochondrial diseases and partly as a complication of failure to thrive | (Hirano et al., 1994),(Yolcu et al., 2014),(Kalkan et al., 2015) |

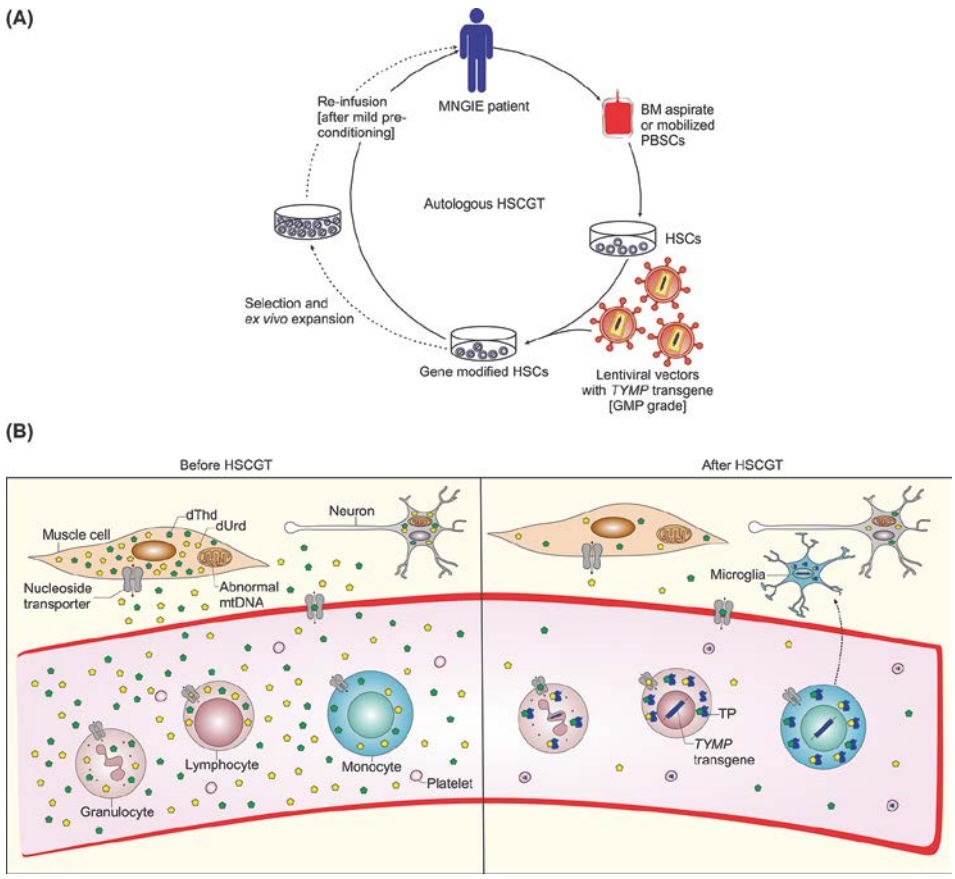


**Figure 2. Schematic representation of autologous hematopoietic stem cell based gene therapy for MNGIE and possible mechanism of biochemical correction by gene modified HSCs. (A)** Autologous bone marrow (BM) aspirates or apheresis of peripheral blood HSCs (PBSCs) after treatment with rh-G-CSF or plerixafor are collected from MNGIE patient. HSCs are *ex vivo* transduced by GMP grade lentiviral vectors containing the human *TYMP* transgene. Before infusion of the transduced cells, MNGIE patients are pre-treated with non-myeloablative conditioning to allow minimal engraftment of gene modified HSCs. (Selection and *ex vivo* expansion of gene modified HSCs allows for transplantation of large numbers of gene modified HSCs to obviate the need for myeloablative pre-conditioning and allows (to some degree) for assessment of safety of the gene modified HSCs prior to transplantation, for example by lentiviral vector integration analysis (reviewed in (Watts et al., 2011). **(B)** The enzyme thymidine phosphorylase (TP) is deficient in all tissues of MNGIE patients, which leads to accumulation of the nucleoside substrates dThd and dUrd and depletion of the nucleotide dCTP and finally mtDNA depletion and deletion (Gonzalez-Vioque et al., 2011). Following transplantation of gene modified HSCs and homing to bone marrow, these cells differentiate into all types of blood cells, LV genome and human *TYMP* transgene are integrated in leukocyte DNA ensuring stable expression of TP. TP catalyzes the chemical reaction which breaks down the nucleosides. This process eventually leads to reduction of systemic nucleosides accumulation. Nucleoside transporters mediate nucleosides transfer via passive facilitated diffusion (ENTs) and active transport (CNTs), the ubiquitous bidirectional ENTs are depicted (Young et al., 2013). In addition, some gene modified HSCs differentiate into monocytes and may migrate to the brain giving rise to microglia which act as a TP reservoir and cross correct the other cells in CNS.

platelets and leukocytes and some other tissues is essential to degrade the excess amounts of dThd and dUrd nucleosides which are secreted into the blood (Lara et al., 2007).

The molecular pathological mechanism in MNGIE involves imbalanced nucleosides and nucleotide pools. Initially, loss of function mutations in *TYMP* gene were identified resulting in reduced TP activity (Nishino et al., 1999) leading to accumulation of access amounts of the nucleoside substrates in blood plasma, urine and almost all tissues (Spinazzola et al., 2002;Valentino et al., 2007). It has been hypothesized that this biochemical imbalance disturbs the equilibrium of intra-mitochondrial deoxyribonucleoside triphosphates (dNTPs) pools (Spinazzola et al., 2002) and hence is responsible for mtDNA depletion, multiple deletions and point mutations associated with MNGIE (Hirano et al., 1994;Papadimitriou et al., 1998) (Nishino et al., 2000;Nishigaki et al., 2003). Therefore, recent studies have addressed the relationship between biochemical and dNTP pool imbalances and subsequent mtDNA abnormalities in MNGIE. *In vitro*, mtDNA point mutations and deletions, similar to those detected in MNGIE patients were reported in cultured HeLa cells after long time culture in the presence of high levels of thymidine in the culture medium. These mtDNA alterations were attributed to expanded levels of deoxythymidine triphosphate (dTTP) and deoxyguanosine triphosphate (dGTP) and reduced levels of deoxycytidinetriphosphate (dCTP) and deoxyadenosine triphosphate (dATP). However, no mtDNA depletion was observed in these HeLa cells (Song et al., 2003). Further investigation revealed that this increase in dTTP, under similar culture conditions, was more pronounced in non-cycling skin and lung fibroblasts leading to depletion in mtDNA in a dThd dose and time dependent manner (Pontarin et al., 2006). Interestingly, mtDNA levels were recovered upon removal of the dThd from the culture medium. In order to understand the influence of metabolites accumulation on the creation of mtDNA alterations, an in organelle experimental model was used. Excess amounts of dThd were responsible for the significant increase in mitochondrial levels of dTTP, together leading to secondary TK2 inhibition mediated reduction of dCTP nucleotides (Gonzalez-Vioque et al., 2011). Subsequent studies confirmed these findings in *in vitro* fibroblast cultures and *in vivo* in the *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mouse model and suggest that the inadequate availability of dCTP accounts for the mtDNA depletion observed in MNGIE (Gonzalez-Vioque et al., 2011;Camara et al., 2014;Torres-Torronteras et al., 2014).

Altogether, these studies demonstrate that indeed it is the nucleoside accumulation and subsequent reduction of dCTP nucleotides, rather than the deficiency of TP *per se*, that accounts for the molecular and phenotypic alterations in MNGIE. An excellent illustration of this observation is the fact that TP expression in skeletal muscles is absent, nonetheless, some but not all MNGIE cases were reported with skeletal muscle mtDNA deletions, histological and oxidative phosphorylation abnormalities (Papadimitriou et al., 1998;Hirano et al., 2004).

When available, although limited, analysis of *postmortem* MNGIE samples is relevant and beneficial to gain knowledge about the molecular and pathological basis of the disease. Severe intestinal dysmotility,

also known as chronic intestinal pseudo obstruction (CIPO), and weight loss are principle presentations of MNGIE. Histopathological analysis of MNGIE gastrointestinal samples revealed depletion in mtDNA and mitochondrial proliferation, and consequently cell atrophy in the muscularis propria layer of the stomach and small intestines (Giordano et al., 2006;Giordano et al., 2008). Additionally, loss of interstitial cells of Cajal and morphologically abnormal muscularis propria and ganglion cells have been reported (Zimmer et al., 2009). On the other hand, the study of brain tissues of 2 MNGIE patients revealed no pathological proliferation of glial cells nor neuronal loss. However, the study suggested a role of TP deficiency in impairment of blood brain barrier, which could contribute to the observed hyperintense T2 signals on brain MRI scans (Szigeti et al., 2004a).

Nucleoside accumulation is detrimental probably during the early course of the disease, because nucleoside clearance did not improve mtDNA content per cell or reduce COX deficient fibers after liver transplantation (De Giorgio et al., 2016). Mitochondrial DNA instability is a hallmark for diseases caused by defective nuclear genes essential for mtDNA replication and repair (such as *PEO1*, *POLG1*,*2*) or maintenance of dNTP pools (such as *ANT1*, *TYMP*) or others involved in mtDNA homeostasis (such as *FBXL4*) (Young and Copeland, 2016). Mutations in *PEO1*, *POLG* and *ANT1* underlie the autosomal dominant form of progressive external ophthalmoplegia (adPEO), a very well characterized mtDNA disorder involving stalling of mtDNA replication (Van Goethem et al., 2001;Goffart et al., 2009). Therefore, stalling of Twinkle helicase or DNA polymerase γ could be a common pathological mechanism underlying mtDNA instability in MNGIE, PEO and mtDNA depletion syndrome (Hirano et al., 2001;Liu et al., 2008).

## | CURRENT TREATMENTS FOR MNGIE

In general, treatment of mitochondrial diseases is mainly based on symptom management and supportive care (Pfeffer et al., 2013). Vitamin and amino acid supplements (Tanaka et al., 1997) and exercise therapy (Taivassalo et al., 1998) aiming to improve mitochondrial functions are recommended for mitochondrial myopathies. Symptomatic management of MNGIE consists of nutritional support (Wang et al., 2015), prevention of infections and pain relief including interventions such as celiac plexus neurolysis and blockage of the splenic nerve (Teitelbaum et al., 2002;Celebi et al., 2006). Since the metabolic and mtDNA abnormalities are attributed to the systemic nucleoside imbalances, clinical interventions focus on direct removal of these metabolites to restore the balance or by introducing the deficient enzyme to reduce the metabolites.

### Hemodialysis and peritoneal dialysis

The first hemodialysis aiming to remove the excess amounts of nucleosides from the circulation was performed in 2002 (Spinazzola et al., 2002) in two MNGIE patients followed by another in 2006. In the first two patients, significantly reduced thymidine levels were observed shortly after hemodialysis, however this effect was

transient as thymidine levels returned to pre-dialysis levels 3 hours after dialysis (Spinazzola et al., 2002). A progressive reduction in Thd levels below the basal levels was observed after repetitive dialysis treatments in the third case (la Marca et al., 2006). A MNGIE patient who had peritoneal dialysis showed an improvement in gastrointestinal symptoms (such as vomiting, anorexia, abdominal pain and diarrhea) during the continuing peritoneal dialysis for three years with body weight gain, although other major symptoms including ocular and neurological abnormalities and brain MRI signals did not change (Yavuz et al., 2007). Another case report noted improvement of the gastrointestinal and neurological symptoms, mainly the mitigation of numbness in the hands, until nucleoside levels increased again 15 months after continuous ambulatory peritoneal dialysis (Ariaudo et al., 2014).

## Enzyme Replacement Therapy (ERT)

Initially, platelet infusions were performed in two MNGIE patients to restore TP enzyme activity in the blood. This approach showed efficient recovery of functional TP enzyme and correction in nucleoside imbalances, however, like dialyses, these improvements were temporary requiring multiple treatment sessions for long-term responses. ERT is a reliable, well-tolerated approach to replace the deficient enzyme in a variety of lysosomal storage disorders including Gaucher, Pompe and Fabry disease and Sly syndrome (Wilcox et al., 2004;Burrow et al., 2007). For MNGIE, approaches were developed to encapsulate TP in order to prolong the half-life of circulatory TP enzyme and reduce the immunogenic reactions. These include polymeric nanoparticles (De Vocht et al., 2009) and erythrocytes as they are permeable and affect the plasma metabolites, such as in adenosine deaminase deficiency (Bax et al., 2000;Moran et al., 2008). The erythrocyte encapsulated TP (EE-TP) concept is under clinical development as an orphan ERT for MNGIE with the first attempt carried out in a MNGIE patient in 2008 (Moran et al., 2008). In this approach, autologous erythrocytes were isolated from patients and loaded with recombinant *E.Coli* TP enzyme *in vitro* via hypo osmotic dialysis. Significant clinical improvements were observed such as the ability to walk and climb and the recovery of sensation and the mitigation of numbness in hands and feet, even after 23 months after termination of multiple cycles of EE-TP (Bax et al., 2013). When using the EE-TP approach, there is a high risk that an immunological reaction is triggered against the bacterial TP, especially if the infusions are repeated several times, although this has not been observed (Levene et al., 2013).

## Orthotopic liver transplantation (OLT)

Liver transplantation is a new ERT strategy for treatment of MNGIE patients. TP protein levels are high in healthy human liver tissues and significantly higher than in bone marrow cells (Boschetti et al., 2014). Recently, OLT was successfully applied in a severely affected, 25-years old MNGIE patient (De Giorgio et al., 2016). Steady state nucleoside balance was observed up to13 months post OLT. Slight improvements in lower limb strength and brain metabolism (reduced lactate levels) and structure (reduced cerebellar mean diffusivity values at diffusion MRI), improved quality of

life scores and nutritional parameters, but not body weight (40 kg), were observed up to six months after OLT. When ileostomy closure was performed, the gastrointestinal (GI) functions and body weight declined at 13 months (37 kg). Therefore, it remains uncertain whether the mild restoration of GI function was due to the decompressive ileostomy, instead of the OLT. In addition, skeletal muscle mtDNA content per cell was slightly increased after OLT. The study suggests that the damage in post mitotic tissues during late stages of the disease is irreversible despite recovery of nucleoside balance. Therefore, biochemical correction should probably be achieved prior to irreversible damage, preferably before the intestinal symptoms appear. Preoperative conditioning for OLT is not required. However, this approach requires matched organ donors (which are limited), involves transplantation related risks and requires long-term immunosuppression which all can further affect the quality of life of the patients.

## Hematopoietic stem cell transplantation (HSCT)

Another possibility to restore TP enzyme activity in the circulation is by HSCT. Recently, a retrospective analysis of all HSC transplanted MNGIE patients between 2005-2011 showed that only nine out of 24 patients were alive up to four years after transplantation. All nine survivors had normalized TP activity in their blood while seven of them showed improved body mass index, gastrointestinal symptoms and peripheral neuropathy. On the other hand, nine MNGIE patients died mainly due to transplant-related causes such as GVHD and graft failure, including recipients of HLA-mismatched unrelated cord blood transplants, while the remaining six patients died of disease progression. The recommendations of this study included transplantation of a sufficient number of cells, because in some patients the graft was rejected, and to consider more closely HLA matched donor cells, because of the large number of GVHD observed in this retrospective study, and to transplant at an earlier age before major organ damage has occurred (Halter et al., 2011;Halter et al., 2015).

The poor physical state of MNGIE patients when they enroll HSCT trials increases the risk for transplantation related complications caused by conditioning regimens and immune-suppressants. Other problems may arise from the drugs used in HSCT which are potentially harmful to mitochondria such as cyclophosphamide (Mariana Ponte Cardoso et al., 2015). Therefore, MNGIE patients are treated with Busulfan and fludarabine prior to HSCT, following the recommendations of MNGIE consensus meeting in 2011 (Halter et al., 2011). For MNGIE patients who develop liver cirrhosis, AHSCT should be contraindicated and OLT would be the treatment of choice (Finkenstedt et al., 2013). Pre-existing liver cirrhosis complicates liver failure which may develop after AHSCT due to multiple factors such as viral infections in immunocompromised recipients or due to hepatotoxic conditioning drugs.

# | PRE-CLINICAL EXPERIMENTAL APPROACHES TOWARDS THERAPY

## Models of MNGIE

*In organello* experiments and human MNGIE fibroblasts were used for highlighting parts of the molecular mechanism of MNGIE (Gonzalez-Vioque et al., 2011;Camara et al., 2014). To study therapeutic interventions, such as gene therapy, *in vivo* models are required. A mouse model was developed by targeted disruption of exon 4 of the *TYMP* gene to generate $Tp^{-/-}$ mice. In contrast to human TP, murine TP degrades both dThd and dUrd; $Tp^{-/-}$ mice were crossed with $Upp1^{-/-}$ to generate the $Tymp^{-/-}Upp1^{-/-}$ mice, which are currently the only relevant *in vivo* animal model (Haraguchi et al., 2002;Lopez et al., 2009). $Tymp^{-/-}Upp1^{-/-}$ mice show increased levels of the purine nucleosides dThd and dUrd in plasma and tissues. Diffuse leukoencephalopathy manifests late during the lifetime of these animals, around the age of 22 months (Lopez et al., 2009). Other symptoms associated with MNGIE, such as decreased motor coordination and gastrointestinal features have not been reported in this mouse model. Brain mtDNA depletion was not consistently found in this mouse model (Lopez et al., 2009;Torres-Torronteras et al., 2011;Camara et al., 2014). Therefore, high doses of exogenous nucleosides were administered to exacerbate the mitochondrial phenotype (Garcia-Diaz et al., 2014), an approach that was rationalized by the lower nucleoside levels in $Tymp^{-/-}Upp1^{-/-}$ mice compared to MNGIE patients. Mice were on an exogenous dThd and dUrd diet for a long time (24 months) before pronounced mtDNA depletion, diffuse leukoencephalopathy and motor abnormalities were observed.

Experimental approaches have been explored for treatment of MNGIE; among which experiments performed by Camara *et al* which suggest that modulation of dNTP metabolism through increasing the availability of dCTP or inhibition of its catabolism can indeed reverse and prevent, at least, dCtd imbalance. A strategy that can be applied for other similar mitochondrial disorders that are caused by altered nucleosides and dNTP metabolism, for example in disorders caused by mutations in *TK2* or *DGUOK* deficiency (Camara et al., 2014).

## Gene Therapy

The $Tymp^{-/-}Upp1^{-/-}$ mouse model has also been used for testing potential curative treatments. A recently investigated strategy is the use of gene therapy. Both lentiviral (LV) and adeno-associated viral (AAV) vector mediated *TYMP* gene transfer have been evaluated in pre-clinical studies for treatment of MNGIE.

### *AAV-mediated liver directed gene therapy*

AAV vector gene therapy has been explored in clinical trials for a variety of inherited and acquired diseases (Naldini, 2015). The main limitation of this approach is the human immune response to AAV capsid, as demonstrated in hemophilia B trials. In one of the first AAV trials targeting the liver, therapeutic levels of coagulation factor IX (FIX) were achieved at a high vector dose ($2\times10^{12}$ vector genomes per kilogram of body weight, vg/kg). Nonetheless, this high vector dose was associated with an early decline of FIX (~8 weeks after treatment)

due to T-cell immunity against AAV capsid antigens eliminating transduced hepatocytes (Manno et al., 2006).

The hybrid vector AAV2/8 with modified molecular configuration (packaged double stranded genome) and improved cassette design (codon optimized hFIX) to enhance transduction and translational efficiency was explored in a hemophilia B trial (Nathwani et al., 2006). Stable FIX expression diminished use of the costly FIX concentrate and importantly, clinical improvement was achieved in a dose dependent manner. The least bleeding episodes were seen in recipients of the highest AAV dose (steady state 5% of normal levels at a single vector peripheral vein infusion of $2\times10^{12}$ vg/kg up to four years) (Nathwani et al., 2014). For MNGIE, an AAV2/8 expressing human *TYMP* under the control of hepatic promoter was used for treatment of *Tymp*$^{-/-}$ *Upp1*$^{-/-}$ mice (Torres-Torronteras et al., 2014). Low AAV doses ($2\times10^{11}$ vg/kg ) were sufficient to reduce nucleoside imbalances to normal levels in liver, skeletal muscle and brain for up to eight months, while higher doses reduced nucleosides below detection levels. However, only at higher doses (>$2\times10^{11}$vg/kg) TP activity was increased in the liver (but not in skeletal muscle or brain).

In light of the clinical data of the hemophilia B trial which shows that clinical improvement was AAV dose dependent (Nathwani et al., 2014), the question for MNGIE is whether or not the low AAV dose would be sufficient to reverse a clinical phenotype beyond biochemical correction. MNGIE mouse studies failed to report any relevant clinical phenotype in *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice, and therefore the potential or required dosage to cure it has not been demonstrated (Torres-Torronteras et al., 2011;Torres-Torronteras et al., 2014). Importantly, upon AAV treatment nucleosides accumulation was not reduced in the intestine of treated mice at the highest dose ($10^{13}$ vg/kg) administered. Since the intestines are heavily affected in MNGIE patients it is important to obtain evidence of correction in this organ. Preclinical studies in hemophilic dogs and non-human primates could predict the therapeutic dose in human trials (Manno et al., 2006;Nathwani et al., 2014) if that is the case for MNGIE too, biochemical correction in the intestine might require improved expression cassettes to enhance protein production, targeting the expression to major affected organs, or the less favorable option of using higher AAV doses (>$10^{13}$ vg/kg). High doses, for example $7.2 \times 10^{12}$ vg/mouse were sufficient to transduce 100% of mouse hepatocytes (Nakai et al., 2005). Such high doses might be required for gene therapy of systemic diseases, i.e. when non hepatic tissues are also affected, as in MNGIE. However, these high AAV doses are likely to cause hepatocellular toxicity, biodistribution to other unwanted organs and shedding of the AAV, enhanced risk of eliciting immunity towards the viral capsid and increased costs of virus production. Immunity against ectopic TP might be an additional concern for MNGIE patients, therefore prophylactic immunosuppression might be required. Additional pre-clinical studies have to address the possibility of an immune response against ectopic TP in previously untreated patients. In addition to increased liver TP activity correlating with vector dose, an unexpected increase in liver dGTP of *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice was observed in a dose depend manner as well, although the consequences of this increase are unknown. Together these findings suggest

that studies into optimal dosing of AAV may be required for clinical application.

Human AAV trials should be carried out cautiously as they can reveal complications that were not observed during preclinical studies. An example is the early decline in FIX expression (Manno et al., 2006) and hepatotoxicity observed in 4/6 recipients of a high AAV dose ($2\times10^{12}$ vg/kg) (Nathwani et al., 2014), due to immunity against AAV capsid. MNGIE patients are often > 12 years and probably have been pre-exposed to AAV and consequently can mount strong immune responses to AAV. Therefore, individuals with neutralizing antibodies to AAV should be excluded from clinical trials to avoid an immune response towards AAV. Another concern is the durability of transgene expression considering the longer lifespan of humans, compared with the animals in preclinical studies, and the potential need for recurrent AAV injections, especially at lower vector doses. In this respect hematopoietic stem cell gene therapy (HSCGT) would provide a preferable option as a single, long lasting intervention method. Additional concerns related to AAV mediated gene therapy include purity of AAV preparations and manufacturing costs (Mingozzi and High, 2013).

### *LV-mediated hematopoietic stem cell gene therapy (HSCGT)*

The encouraging therapeutic outcomes and favorable safety profile renders LV-HSCGT an attractive therapeutic approach for a variety of hereditary metabolic disorders (Wagemaker, 2014), and is potentially advantageous over AHSCT for certain selected diseases (Naldini, 2015). Proof of concept of HSC gene therapy was obtained in *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice (Torres-Torronteras et al., 2011) using a phosphoglycerate kinase promoter driving native human *TYMP* cDNA and a GFP reporter in hematopoietic cells resulting in biochemical correction in peripheral blood (Torres-Torronteras et al., 2011). More recently, we developed clinically applicable LVs that carry human *TYMP* cDNA, and demonstrated long-term biochemical correction in *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice at low vector copy number (VCN). Our data demonstrates the feasibility to further develop clinical protocols for HSCGT for MNGIE (Yadak et al., 2015). Similar results in a long-term follow up of 20 months confirms the correction of biochemical imbalances which was maintained at low vector copy number and chimerism (Torres-Torronteras et al., 2016).

In HSCGT for MNGIE, HSCs are isolated from MNGIE patients, transduced *ex vivo* by LV vectors carrying a functional copy of *TYMP* and infused back into the patient (**Figure 2A**). The newly formed HSCs and its progenitors produce TP which catabolize the excess amounts of nucleosides (**Figure 2B**). Since the patient's own stem cells are used, GVHD is not a concern. However, mild prophylactic immunosuppression maybe required to prevent possible immune reaction against the TP transgene.

Myeloablative pre-conditioning might be necessary for high levels of engraftment in other metabolic disorders, such as metachromatic leukodystrophy (MLD), due to lack of selective advantage of gene modified cells. In particular, busulfan myeloablative conditioning is used in MLD patients for depletion of endogenous microglia and mobility of gene modified monocytes through the blood brain barrier (BBB) (Capotondo et

al., 2012). The contribution of gene modified microglia to correct biochemical imbalances has never been explored in MNGIE. However, murine gene therapy studies using LV and AAV vectors implicate reversal of nucleoside imbalance at low or possibly no increase in brain TP activity (Torres-Torronteras et al., 2014;Torres-Torronteras et al., 2016). In liver directed AAV2/8 gene therapy, it is not expected that brain cells will be transduced. In HSCGT, gene modified monocytes are expected to migrate to brain and differentiate into microglia. Nonetheless, the results of the HSCGT MNGIE mouse study do not rule out the potential that gene modified microglia can contribute to correction of brain biochemistry and phenotype, although this might not be necessary if ectopic expression outside the brain is high enough. When transduction efficiency is high enough, significant TP activity can be measured in the brain, indicating that transduced microglia might reside in the brain after long-term follow-up. To that end, two potential mechanisms might act synergistically to normalize the brain nucleoside levels, a systemic ectopic source and one local contribution of gene-modified cells **(Figure 2B)**.

Potential options and future research for application in MNGIE patients include alternative conditioning strategies to obviate the cytotoxicity related to myeloablative conditioning and strategies to enhance the quality of infused gene modified HSCs. One approach is to mobilize endogenous HSCs into peripheral blood in order to create (space) in the bone marrow for the infused donor HSCs to engraft (Chen et al., 2006). Human granulocyte colony stimulating factor (G-CSF) was sufficient in immunocompromised mice (Huston et al., 2014), probably due to the selective advantage of the gene modified cells, however more stringent agents might be required in normal immunocompetent mice. A possibility is G-CSF in combination with the more potent HSCs mobilizer plerixafor (a specific CXCR4 antagonist) or the selectins inhibitor fucoidan. Such regimens probably require additional mild chemotherapeutics, in particular if the gene corrected TP-expressing HSCs lack selective growth advantage to overcome host cells. These HSCs mobilizers act via different mechanisms, therefore parameters such as the optimal dose and time frame for transplantation after mobilization need to be established in relevant pre-clinical models. Alternatively, targeting specific endogenous hematopoietic populations might reduce the off-target toxicity related to the common non-specific conditioning (Aiuti and Naldini, 2016). Examples include inhibiting c-kit, a HSC tyrosine kinase cell surface antigen (Xue et al., 2010) and the recently developed immunotoxin against hematopoietic stem cells (CD45-SAP) (Palchaudhuri et al., 2016).

Strategies such as *ex vivo* expansion of gene modified HSCs can improve the quality of the infused gene modified cells and enhance the outcome of gene therapy (Watts et al., 2011). In particular when combined with additional approaches to enrich for HSCs, preserve stemness of- and enhance homing and engraftment ability of gene modified HSCs (Psatha et al., 2016). Ultimately, this approach combined with improved mild pre-conditioning protocols, could benefit patients in poor health condition at transplantation, such as in MNGIE patients.

A risk of HSC gene therapy is insertional mutagenesis. The first HSC gene therapy

trials used gammaretrovirus (γ-RV) based vectors for treatment of X-linked severe combined immunodeficiency (SCID-X1) (Gaspar et al., 2004;Hacein-Bey-Abina et al., 2010), adenosine deaminase (ADA-SCID) (Aiuti et al., 2002), chronic granulomatous disease (CGD) (Ott et al., 2006) and Wiskott-Aldrich syndrome (WAS) (Boztug et al., 2010). Although efficient correction of immunodeficiency was achieved in most patients in SCID-X1, CGD and WAS trials, lympho-proliferative disorders (Hacein-Bey-Abina et al., 2003a;Hacein-Bey-Abina et al., 2003b;Hacein-Bey-Abina et al., 2008;Howe et al., 2008;Braun et al., 2014) and myelodysplasia (Stein et al., 2010) developed secondary to γ-RV vector integrations within or nearby proto-oncogenes. In addition to the preferred integration profiles over γ-RV vector (Deichmann et al., 2007;Gabriel et al., 2012;Cattoglio et al., 2007), LV efficiently transduce non-cycling primitive HSCs and under minimum culture conditions (Naldini et al., 1996;Guenechea et al., 2000). Therefore, attention was focused on development of LV as a relatively safer approach, leading eventually to development of third-generation self-inactivating HIV derived-vectors (SIN-LV) (Dull et al., 1998;Zufferey et al., 1998). Several pre-clinical studies indicate the reduced genotoxicity of SIN-LV vectors compared with γ-retroviral vectors, in particular SIN-LV common integration sites (CIS) revealed no preference of integration near proto-oncogenes (Montini et al., 2006;Modlich et al., 2009;Romero et al., 2013;Zhou et al., 2013;Biffi et al., 2011). Since then, SIN-LV vectors have been applied successfully in ongoing clinical trials for a variety of metabolic (Cartier et al., 2009;Biffi et al., 2013) and immunodeficiency disorders (Aiuti et al., 2013), and no adverse events have yet been reported in these trials. Moreover, the therapeutic benefits without toxicity related to transgene expression and biosafety of SIN-LV vectors has been further validated through a growing body of recent preclinical studies supporting the initiation of clinical trials, for example for β-thalassemia (Negre et al., 2015) and mucopolysaccharidosis I disease (Visigalli et al., 2016).

Furthermore, selective advantage for growth and differentiation conferred by the therapeutic transgene expression increases the potential risk for proliferative disorders, this was reported in some immunodeficiency conditions (Aiuti and Roncarolo, 2009). For metabolic disorders, for instance lysosomal storage disorders , however, most studies show that enzyme positive cells have no selective advantage (Bernardo and Aiuti, 2016), which is most likely the case in MNGIE as well. To improve safety, technologies such as *ex vivo* expansion of gene modified HSCs may permit for safety assessment (to some degree) prior to transplantation, by analysis of LV integration sites (Watts et al., 2011) **(Figure 2A)**.

### *AAV mediated GT or HSCGT?*

Regardless of the type of viral vector used for gene therapy, the chosen strategy should provide long-term expression of the gene of interest without side effects in the host. It is important to apply a well-defined vector dose that is sufficient to reverse the biochemical and nucleotide imbalance without any potential side effects. In particular, abnormal overexpression of the TP enzyme is detected in different tumor types, including non-small cell lung-, colorectal-, breast-, gastrointestinal-, and hepatic cancers (Koukourakis et al.,

1997;Mori et al., 2000;Ikeguchi et al., 2001;Nakayama et al., 2005;Mitselou et al., 2012) and correlates with a worse prognosis in colorectal cancer patients (Takebayashi et al., 1996). Besides, disturbance of dNTP pools can be a trigger for cell cycle arrest and apoptosis (Oliver et al., 1996) (Kumar et al., 2010).

The medical condition of the patient can also influence the choice of the vector system for clinical application. For terminally ill patients, the AAV approach could be most suitable to avoid the risks associated with the pre-conditioning for transplantation in autologous HSC gene therapy or if a suitable HSCs donor for AHSCT is lacking.

## | CONCLUDING REMARKS

The lack of mitochondrial histone protection, the limited repair capacity and oxidized dNTPs contributing to mismatch errors (Alexeyev et al., 2013) all make mitochondria more susceptible than nuclear DNA to mutagenesis. It has become evident that it is the systemic accumulation of nucleosides in MNGIE (Di Meo et al., 2015) that causes imbalances in mitochondrial dNTP pools. However, the mechanism by which it causes mtDNA alterations is still unknown. Although the current treatments focus on restoration of TP enzyme activity and/ or elimination of accumulating metabolites, further understanding of cellular mechanisms involved in maintenance of mtDNA integrity and copy number can provide targets for clinical intervention for MNGIE and possibly other mitochondrial disorders.

Platelet infusions, hemato/peritoneal dialysis and erythrocyte encapsulated TP enzyme replacement therapy could be used to provide biochemical correction. AAV gene therapy and lentiviral HSCGT are potential curative options as evidenced by the promising pre-clinical results in *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice . OLT is a promising emerging treatment and should currently be the treatment of choice for MNGIE patients with pre-existing liver failure. Allogeneic HSCT has risks of graft failure, GVHD and conditioning-related toxicity. Milder conditioning may be applicable in HSCGT, and treatment should preferably be applied at an early age. Novel strategies are being explored to improve the safety and efficiency of viral based gene therapy, ultimately for MNGIE patients as well. These include strategies to enhance transduction, improve engraftment of gene modified HSCs and limit transplantation related toxicity, and others to overcome the limitation of AAV capsid triggered immunity by means of novel serotypes and improved transcription cassettes.

MNGIE patients should receive suitable treatment promptly before permanent damage occurs, which can be challenging, as MNGIE patients are often diagnosed late during disease progression in a poor health condition. Because TP activity and nucleoside levels can be routinely measured in blood samples, MNGIE should be considered to be included in newborn screening programs, similar to other (neuro) metabolic disorders for early diagnosis and treatment (Carlson, 2004;McHugh et al., 2011).

## LIST OF ABBREVIATIONS

AAV: adeno-associated virus; ADA-SCID: adenosine deaminase-severe combined immunodeficiency; AHSCT: allogeneic hematopoietic stem cell transplantation; ANT-1: adenine nucleotide translocase type 1; BBB: blood brain barrier; CGD: chronic granulomatous disease; CIPO: chronic intestinal pseudo obstruction; CIS: common integration site; CNT: concentrative nucleoside transporter; CPEO: chronic progressive external ophthalmoplegia; dATP: deoxyadenosine triphosphate; dCTP: deoxycytidinetriphosphate; dGTP: deoxyguanosine triphosphate; DGUOK: deoxyguanosine kinase; dTTP: deoxythymidine triphosphate; dNTP: deoxyribonucleoside triphosphates; dThd: thymidine;dUrd: deoxyuridine; ECGF1: platelet-derived endothelial cell growth factor; EE-TP: erythrocyte encapsulated TP; ENT: equilibrative nucleosides transport; ERT: enzyme replacement therapy; FBXL4: F-box and leucine-rich repeat protein 4; FIX: coagulation factor IX; G-CSF: granulocyte colony stimulating factor; γ-RV: gammaretrovirus; GVHD: graft versus host disease; HSCT: hematopoietic stem cell transplantation; HSCGT: hematopoietic stem cell gene therapy; LHON: Leber's hereditary optic neuropathy; LV: lentivirus; MDS: mitochondrial DNA depletion syndrome; MNGIE: mitochondrial neurogastrointestinal encephalomyopathy; MELAS: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; NT: nucleoside transporter; OLT: orthotopic liver transplantation; PEO: progressive external ophthalmoplegia; POLG: DNA polymerase subunit gamma; RRM2B: Ribonucleotide Reductase M2 B; SCID- X1: X-linked severe combined immunodeficiency; SIN-LV: self-inactivating LV; TK2: thymidine kinase 2; TYMP: thymidine phosphorylase; WAS: Wiskott-Aldrich Syndrome.

## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

RY framed the structure of the review, analyzed the literature and wrote the manuscript; PS, MW participated in the literature analysis; NP, IF participated in the literature analysis and supervised the writing.

All authors discussed the topic and provided intellectual feedback to the article.

All the authors read and approved the manuscript.

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# General discussion

7

# LENTIVIRAL VECTOR GENE TRANSFER INTO HEMATOPOIETIC STEM CELLS

Hematopoietic stem cell gene therapy (HSCGT) has been successfully used in clinical trials for a wide range of inherited hematological, metabolic, and immune deficiency disorders.[1] Recently, the first *ex vivo* HSCGT drug (Strimvelis) was approved for marketing in Europe for the treatment of adenosine deaminase severe combined immunodeficiency (ADA-SCID). The product is made of autologous hematopoietic stem cells (HSCs) transduced with a gammaretroviral (γ-RV) vector containing ADA, therefore an excellent personalized treatment of ADA-SCID patients for whom a matched donor is lacking. Due to the highly proliferative capacity of hematopoietic stem and progenitor cells (HSPCs), RVs have been employed in HSC gene transfer protocols for their capacity to integrate into the host's genomic DNA and propagate transgene expression into progenitors and mature cells. Pre-clinical studies resulted in sufficient proof of concept [2,3] to move towards clinical trials for primary immunodeficiencies using γ-RV vectors. These trials showed the efficacy of this gene therapy approach demonstrating phenotypic correction and clinical improvements following the restoration of the cellular, and in some, also the humoral immunity.[4,5] However, a large proportion of the X-linked SCID and Wiskott Aldrich γ-RV trials reported hematological malignancies related to the upregulation of proto-oncogenes close to the vector insertion sites.[6-11] Therefore, efforts were directed towards the development of third generation self-inactivating (SIN) lentiviral (LV) vectors as an alternative vector system for reducing the risk of insertional oncogenesis.[12]

Another advantage of using LV vectors is that the proliferation of HSPCs during gene transfer is not a requirement, in contrast to γ-RV vectors.[13] The current protocols using RV (both γ-RV and LV) employ two rounds of transduction, high vector doses (MOI 100), and relatively high concentrations of multiple cytokines.[14,15] These conditions, however, could affect the efficacy and safety outcomes of HSCGT, by increasing the risk of insertional mutagenesis of the genes involved in cell-cycling and by compromising the long-term repopulation capacity of gene modified HSCs. Our findings show efficient gene transfer into HSPCs during a short transduction time by increasing the target cell density with a proportional increase of LV particles. Addition of thrombopoietin (TPO) alone was sufficient in maintaining the *in vivo* repopulation capacity and ensuring efficient transduction at the same time. Our evaluation of human HSPCs was limited to *in vitro* assessments, and further experiments might be required to estimate the efficacy of prolonged gene marking and repopulation capacity through transplantation into the humanized mouse model.[16] Additionally, of interest is the application of this protocol in mouse models of human diseases to evaluate the contribution towards phenotypic correction; performance of the transductions in microfluid-based transduction system, wherein the probability of infection is increased;[17] and combination with prostaglandin E2 stimulation leading

to effective LV transduction of HSCs during short culture time (less than 38hrs).[18]

These advancements at generating a clinically viable and feasible protocol for LV transduction of HSPCs could be combined with strategies to enhance the expansion of gene modified HSPCs. *Ex vivo* expansion would support the maintenance of the (stemness) of the gene modified HSPCs, enable selection of HSPCs with integrated LV vector copies and transgene expression prior to transplantation, and the evaluation of LV-integration site profiles. In that regard, biomolecules such as neurotrophic factors represent novel candidates for enhancing the expansion and survival of HSPCs, [19] while the pyrimidoindole derivative UM171 was recently successfully employed for the *ex vivo* expansion of LV transduced human mobilized peripheral blood HSCs with long term repopulation capacity.[18] The therapeutic outcomes can be further enhanced by the integration of approaches aiming at improving the homing and engraftment of gene modified HSPCs. These strategies include inhibition of CD26[20] peptidase that negatively affects the levels of the chemokine stromal cell-derived factor 1 (SDF-1) and priming of HSCs with prostaglandin E2 prior to transplantation.[18,21,22]

## HSCGT: an efficient and safe treatment for MNGIE?

Allogeneic HSCT for treatment of MNGIE is associated with a high mortality rate and the therapeutic outcomes are transient and limited.[23,24] As a result of the encouraging therapeutic outcomes and incremental improvements related to LV vector design, HSCGT holds promise as a safe and effective treatment for multiple life-threatening diseases, including MNGIE.

Development of a LV vector-mediated HSCGT protocol for the treatment of MNGIE would require ***(i)*** a clinically relevant mouse model, ***(ii)*** a clinically applicable LV vector, ***(iii)*** assessment of therapeutic outcomes, beyond correction of the biochemical phenotype, and ***(iv)*** assessment and minimizing the risks related to pre-transplant conditioning, transgene expression and LV vectors.

The studies performed in this thesis address these points, but clinical translation requires additional studies to provide HSCGT as a cure for MNGIE patients.

***(i)*** The generation of *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice was instrumental to enhance the development of alternative treatments to cure MNGIE patients.[25] *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice exhibit a biochemical phenotype similar to MNGIE patients (elevated dThd and dUrd), however, only ≥18 month old mice demonstrate mild neurological phenotypes. The lack of other relevant similarities to MNGIE such as alterations of mitochondrial DNA (mtDNA) and pronounced motor dysfunctions, limits the evaluation of the therapeutic outcomes beyond the biochemical phenotype.[26-28] To enforce these phenotypes the nutritional diet can be changed, consisting of increased dThd and dUrd amounts, but mice still need to be maintained for very long periods (up to 24 months).[29] These limitations challenged the suitability of *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice to model MNGIE. We therefore carefully characterized the brain and intestinal phenotypes at an earlier age to evaluate HSCGT efficacy. First, we carefully examined the neurological aspects of *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice which confirmed the previously reported white matter changes

in brain MRI and histology,[25] but at an earlier age, and additionally demonstrated an abnormal phenotype of brain astrocytes. Further experiments are required to elucidate the nature of the cellular and molecular changes observed in brain astrocytes morphology and the altered mtDNA replication, and how that relates to unbalanced nucleosides levels. In this regard, experiments to evaluate ion-water homeostasis are relevant. Specifically, we observed an increase in the thickness of the astrocytes processes in *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice. This is similar to megalencephalic leukoencephalopathy with cysts (MLC) mice models (*Mlc1*-null and *Glialcam*-null mice). These studies provide evidence that defective astrocytic fluid volume regulation underlies the pathomechanism of MLC. [30,31] Future studies could be performed to investigate the status of volume regulated anion channel (VRAC) currents and regulated volume decrease in astrocytes of *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice. Additionally, we report abnormalities in the myogenic and neurogenic compartments of the intestine of *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice. However the mice did not develop profound cachexia, unlike MNGIE patients, perhaps due to *ad libitum* feeding, or that the mice are double knock out for two genes unlike in MNGIE patients, or the physiological differences between mice and man. Nonetheless, the intestinal pathology of *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice occurs at an early age and is similar to the pathology in MNGIE patients. Therefore, testing potential treatments in the mouse model may still predict the therapeutic response in MNGIE patients.

***(ii)*** Our results indicate that the developed therapeutic LV vectors (PGK-TP(co) could efficiently correct MNGIE phenotypes with the following minor modifications: **(a)** The physiological promoter (hPGK) led to lower levels of TP expression which were sufficient for phenotypic correction, without the increased risk of transactivation of nearby oncogenes as compared with the strong promoter of the spleen focus forming virus (SF); **(b)** at low VCN/cell (median ≤ 2.3, MOI 10); and **(c)** at moderate levels of engraftments (median, 77 %, PGK-TP(co)). Altogether, these results indicate that the therapeutic LV-PGK-TP and TPco vectors mediate efficient correction, thereby abrogating the necessity for further enhancement of the vector performance. Although codon optimization of *TYMP* did not enhance *TYMP* transcription or protein levels, unlike in the case of other disease models,[32,33] other algorithms might be investigated to still improve protein production per VCN.

In order to control transgene expression, enhance the biodistribution of- and improve the reconstitution of- transduced hematopoietic cells, numerous modifications could be implemented. Cell specific targeted transgene expression could be achieved through the incorporation of cell specific promoters or enhancers such as GP1BA, ITGA2B, and PF4 megakaryocyte-specific gene promoters [34] to selectively drive moderate levels of *TYMP* expression in platelets, a rich source of TP in healthy people. Tropism of LV vectors can be altered through modifications of LV envelopes to target long-term repopulating stem cells, or the expression restricted to certain cell types via miRNA de-targeting (reviewed in Goyvaerts C, *et al.* 2013).[35] This would allow for strong and prolonged expression levels in target stem cells, without the toxicity on nontarget cells. Alternatively, targeted gene editing of HSCs

with artificial endonucleases such as Zinc-Finger Nucleases (ZFNs), Transcription Activator Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated (CRISPR/Cas) could reduce the risk of insertional oncogenesis through the direct integration into pre-determined genomic loci. In this regard, protocols are developed for efficient gene targeting of HSCs by using ZFN, augmented with adapted culture conditions.[22] The delayed culture timing prior to gene targeting and the presence of StemRegenin 1 and prostagandin E2 to preserve stemness permitted for efficient targeted integration in human HSCs with long term repopulation in immunodeficient mice. The functionality of this approach was proven by targeted integration of the corrective cDNA into *IL2RG* in HSCs from a SCID-X1 patient. 3-11% GFP+ were detected in the BM CD34+ subpopulations isolated from the patient after targeting for *IL2RG* correction. For MNGIE, probably higher efficiency are required, since *TYMP* gene corrected cells, in contrast with *IL2RG* corrected cells, might not confer a selective advantage.

In contrast to ZFNs, TALENS targeting the same site of CCR5 were reported to have a reduced off-target activity.[36] TALENs were successfully used for targeting the human β-globin locus; i.e. correction of the pathological mutations in iPSCs from patients of β-thalassemia[37] and sickle cell anemia,[38] to express normal b-globin in the differentiated hematopoietic progenitors and erythroblast. This is probably also feasible for correction of *TYMP* as well. The CRISPR/Cas9 system can be used for efficient gene editing of primary murine and human HSPCs,[39] or patient-derived induced pluripotent stem cells (iPSCs). In this approach, CRISPR/Cas9 is applied for correction of the disease underlying mutation in patient-derived iPSCs, which are differentiated afterwards to, for instance, hematopoietic progenitor cells, for transplantation in patients. The majority of lysosomal storage diseases are candidate targets for treatment with this approach, because of the monogenic mutations and the ability of gene corrected cells to excrete the deficient enzyme.[40] These advancements and progress towards clinical trials (e.g. treatment of HIV infection[1], and mucopolysaccharidosis I and II[2]) render therapeutic gene editing an attractive treatment option, feasible for application in MNGIE patients as well. However, several limitations need to be considered prior to the clinical application of iPSCs. These include the large-scale manufacturing steps such as the genetic re-programming and *in vitro* expansion, increased incidence of deletions or changes in DNA copy number and thereby tumorigenesis, which is also underlined by the use of integrating viruses and oncogenes, and risks of immune response or graft rejection. Furthermore, translation of gene editing strategies into clinical settings requires further enhancements of the delivery, efficiency and specificity of the gene correction machinery. First, *in vivo* gene editing is usually performed with AAV,[41] which imposes challenges including off-target editing related to the constitutively expressed nucleases, and immunity against

**https://clinicaltrials.gov/ (2017) [1](NCT01252641), [2] (NCT02702115, NCT03041324)**

certain serotypes. On the other hand, for *ex vivo* gene targeting of HSCs, transient expression of the nuclease is sufficient and can be achieved through electroporation or non-integrating LV vectors.[22,39] In order to avoid excessive expression of nucleases and off-target editing integrating LV vectors are not preferred. Second, the efficiency of targeted gene correction could be enhanced by modulation of double strand break pathways, which are dependent on the target cell cycle. Modulation of *ex vivo* cell culture conditions could be useful to control the cell cycle.[22]

In addition, several approaches can be applied in order to achieve above normal *TYMP* expression levels (as discussed in ***iii***) including the targeted integration of an expression cassette with a strong promoter in a pre-selected safe harbor such as AAVS1, or fusion of the open reading frame of TYMP with the mRNA of a highly expressed endogenous gene such as CD45 in HSPCs. As in the case for the treatment of hemophilia B, *in vivo* genome editing by using ZFNs and AAV2/6 was successfully used for the insertion of a therapeutic copy of human factor 9 into the albumin locus in liver hepatocytes, leading to the production of therapeutic levels of human factor 9 (>1% of normal) in mice and non-human primates.[42]

Finally, off-target gene editing and constitutively expressed nucleases could result in undesired alterations of cancer-related genes or lead to loss of function, therefore the specificity of CRISPR/Cas9 targeting can be enhanced by selection of the nucleases used,[43] or by using truncated guide RNA, guide RNA extension, Cas9 nickases, RNA-guided FokI–dCas9 nucleases, or engineered Cas9. [44]

***(iii)*** Above-normal TP activity was required for restoration of neurological and intestinal phenotypes and was achieved with LV-PGK-TP(co) at a moderate LV dose (MOI 10). This is similar to findings in other metabolic disorders, where above-normal expression of the therapeutic enzyme led to correction of disease phenotypes which are otherwise not responsive to traditional HSCT. This includes the amelioration of neurological phenotypes in mucopolysaccharidose II [45] and globoid cell leukodystrophy mice[46] (reviewed in Biffi A, *et al*. 2017).[47] Only the highest dose used in our studies in *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice, an MOI of 10, resulted in above-normal levels of TP activity in blood (range, 68-188 nmol/h/mg protein), was just sufficient at normalizing intestinal TP activity and rescued the pathology after HSCGT, while HSCT in MNGIE patients did not rescue the intestinal pathology despite biochemical correction as demonstrated in **Chapters 4 and 5** and by Halter *et al.* (range, 262-1285 nmol/h/mg protein).[23] Graft versus host disease or graft rejection following allogeneic HSCT, timing of treatment (HSCGT was administered at the same age when the intestinal pathology was minor, versus MNGIE patients who are usually treated at a late stage of the disease), or interspecies differences could also explain the contrasting responses of human and mice to treatments.

In contrast to using LV-mediated HSCGT, above-normal TP levels in blood were not achieved through AAV2/8-mediated liver directed gene therapy in *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice, even with high vector dose (>2×10$^{11}$ vg/kg). Importantly, nucleoside accumulation was not reduced in the intestine of these mice.[27] AAV gene therapy for MNGIE is an attractive treatment option because it does not require

pre-conditioning or a suitable HSCs donor. However, compared with clinical trials for hemophilia B, in which a small proportion of transduced hepatocytes is sufficient to convert a severe patient into a moderate phenotype, it is speculated that an effective treatment for MNGIE would require an improved expression cassette, targeted expression in affected organs or a higher AAV vector dose. A higher dose increases the risk of eliciting immunity towards the viral capsid and liver toxicity. The pros and cons of AAV- and LV-based gene therapy approaches are discussed in more detail in **Chapter 2**.

In addition to the above-normal TP levels, the timing of treatment is critical for the resolution of the intestinal phenotypes. HSCGT was administered prior to detectable pathological appearance (at 2 months of age), whereas MNGIE patients receive HSCT usually when symptomatic (i.e. after development of a clinical phenotype). To that end, early diagnosis through genetic screening of newborns is critical. Nonetheless, vector and transplanted gene modified cell dose escalation experiments involving a large group of mice is required to establish the minimal dose sufficient for phenotypic correction and without cellular or molecular toxicity before translation into a clinical protocol for treatment of MNGIE patients.

Moreover, albeit correction of the biochemical, neurological, and intestinal phenotypes we were unable to evaluate the contribution of HSCGT to recovery of apparent clinical phenotypes such as motor function and cachexia, due to their absence in the *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice. *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice fed with a diet consisting of dThd and dUrd in order to induce the clinical phenotypes [29] could be used to assess the effects of HSCGT on recovery of apparent phenotypes such as motor dysfunction. ***(iv)*** Adverse events could occur that are related to toxicity of the pre-transplant conditioning, transgene over expression or immunity against the transgene product, or genotoxicity of integrated LV vectors. In this thesis, we addressed these points in relation to HSCGT for MNGIE. Prior to HSCT, a relatively mild conditioning consisting of busulfan and fludarabine or in combination with anti T cell antibodies, is usually applied. [23] However, an alternative mild conditioning could be preferred for treatment of MNGIE patients who are usually in a very poor condition. Novel non-cytoreductive conditioning approaches have been successful in preclinical studies, including the administration of HSC mobilizing cytokines such as human granulocyte colony stimulating factor,[48] or antagonists of endogenous HSC markers such as anti c-kit [49] or anti CD45.[50] Our results show that following sub-lethal total body irradiation of *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice, moderate levels of engraftment were sufficient for disease correction. Therefore, application of the novel non-cytoreductive pre-transplant conditioning may be adequate to achieve sufficient levels of donor cell chimerism for disease correction, and avoid toxicity and further deterioration of mtDNA, in particular when combined with the advancements of gene transfer protocols and the *ex vivo* expansion. In order to assess any potential phenotoxicity related to *TYMP* over expression, further in depth experiments are required. In this regard, it is important to evaluate if HSCGT sufficiently restores the depleted dCTP levels and investigate any potential negative effects of TP over expression on dNTPs pool homeostasis,

mtDNA replication, cell cycle arrest, and apoptosis.[51,52]

The results of this thesis indicate that HSCGT for MNGIE can be performed with low risks of *in vivo* mutagenesis or integration nearby oncogenes. Follow up of primary and secondary recipients of LV-PGK-TP(co) for 22 months revealed no detectable hematological abnormalities and the LAM-PCR demonstrated benign vector integration patterns. No differences in the frequency of integration near oncogenes were observed between the control and gene therapy treated groups, or classified based on the promoter (PGK vs. SF) or transgene (therapeutic vs. GFP) used and were also similar to those obtained for different inherited diseases such as in *Il2rg*$^{-/-}$ and *Gaa*$^{-/-}$ mice. Two secondary recipients in the PGK-TP-GFP treatment group developed B cell lymphoma; the clone contained a single dominant integration site in gene *Zfp207*. *Zfp207* is required for proper chromosome alignment[53] and therefore, interruption by for instance, a LV insertion could have augmented oncogenesis. This requires further experiments to elucidate the role of *Zfp 207* as a potential tumor related gene. For example, DNA and RNA sequencing can be performed in order to profile altered signaling pathways or changes in the mitotic checkpoint protein Budding Uninhibited by Benzimidazoles 3 (BUB3), the main target for *Zfp207*, as well as functional studies that entitle knockdown of *Zfp207 in vitro* or *ex vivo* to determine the effect on cycling HSCs, and to determine its role in the contribution of oncogenesis.

In conclusion, LV vector-mediated HSCGT for MNGIE patients is a feasible treatment option which can successfully reverse the biochemical, neurological, and intestinal phenotypes in *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice without apparent toxicity. Clinical application of HSCGT for the treatment of MNGIE patients would require further experiments to assess the minimal LV dose for the correction of clinical phenotypes such as motor function or cachexia, and additional evaluation of potential toxicity related to *TYMP* over expression. Future advancements could involve strategies to direct and control transgene expression in target cells, and strategies for improving the HSCGT procedure such as enhanced LV transduction of HSPCs and *ex vivo* expansion and selection of gene modified cells prior to transplantation, in addition to the application of non-cytoreductive preconditioning.

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# Appendix

# SUMMARY

Hematopoietic stem cell gene therapy (HSCGT) is an attractive treatment option for a wide range of disorders. The initial clinical trials for X- linked severe combined immunodeficiency (SCID-X1) and ADA-SCID applied γ-RV vectors successfully, but hematological malignancies occurred in SCID-X1 due to vector integration and transactivation of nearby oncogenes. Therefore, efforts were directed towards finding alternative viral vector systems leading, eventually, to the development of the new 3rd generation self-inactivating (SIN) lentiviral (LV) vectors with enhanced efficiency and benign integration profiles.

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is currently treated with allogeneic hematopoietic stem cell transplantation (allogeneic HSCT). Allogeneic HSCT successfully reverses the biochemical phenotype. However, HSCT is associated with high mortality rates, requires the availability of matched donor hematopoietic stem and progenitor cells (HSPCs) while knowledge about the long term therapeutic outcome is limited. LV-mediated HSCGT uses autologous cells, may require lower pre-conditioning regimens and is expected to provide long-term systemic disease correction as discussed in **Chapter 2**.

In **Chapter 3**, we optimized the *ex vivo* culture conditions of LV vector mediated gene transfer into HSPCs to achieve efficient gene marking within a short transduction time period, with a minimal number of integrated vector copies, and minimal cytokine additions. The results indicate the feasibility to improve gene transfer by increasing the density of mouse, rhesus, or human HSPCs target cells. Additionally, overnight transduction of mouse and human HSPCs in stem cell medium with addition of thrombopoietin (TPO) only enhanced transduction sufficiently for *in vivo* purposes. The addition of the growth factors SCF, TPO, Flt3-L at relatively low concentrations provided the most efficient gene transfer (> 60% GFP marking) while maintaining progenitor and stem cells repopulation capacity. Altogether, these findings open intriguing possibilities for overcoming the limitations of the current gene transfer protocols: an enhanced efficiency and improved safety of gene correction, minimal *ex vivo* manipulation during a short culture period, and the added economic value of using small amounts of clinical grade lentiviral vectors and cytokines.

**Chapters 4 and 5** focus on the evaluation of the efficiency and safety of LV vector-mediated HSCGT as a treatment for MNGIE in *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice. Therapeutically applicable SIN LV vectors were used containing the physiological human phosphoglycerate kinase (hPGK) promoter for ubiquitous expression of native or codon optimized human TYMP cDNA (PGK-TP or PGK-TPco, respectively). LV-SF-TPco bearing the strong spleen focus forming virus (SF) promoter was used for safety evaluation to highly express TP, and to assess potential genotoxicity. The results in **Chapter 4** demonstrated persistent TP activity and clearance of nucleosides in the brain of treated *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice at relatively low vector copy numbers (PGK-TP(co): MOI 10, range; 0.2-3.6 VCN/cell). Here, we showed that this resulted also in reversal of the phenotypic alterations apparent in brain astrocytes of

*Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice, as well as reduction of white matter edema demonstrated by brain MRI and immunohistological analysis. Long term follow up of recipients of HSCGT revealed no side effects from the procedure: ***(i)*** Graft failure or toxicity related to sub-lethal total body irradiation was not observed. ***(ii)*** Survival curves of mice in the PGK-TP(co) treatment groups did not differ significantly from controls. ***(iii)*** LV- related insertional mutagenesis or hematopoietic transformation were not observed after a total of 22 months follow up of primary and secondary recipients of HSCs transduced by therapeutic LV vectors PGK-TP(co). ***(iv)*** LAM-PCR and sequencing demonstrated a polyclonal integration pattern and no bias towards integration near or selection of proto-oncogenes.

Gastrointestinal manifestations are prominent and the main cause of death among MNGIE patients. In **Chapter 5** we examined the pathology of the small intestine of MNGIE patients and *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice and the impact of allogeneic HSCT or gene therapy, respectively. Our findings confirmed the previously reported atrophy of the muscularis propria and absence of interstitial cells of Cajal in MNGIE patients. HSCT did not resolve these pathological characteristics in the short period studied post transplantation. This might explain the repeatedly reported persistent gastrointestinal manifestations after treatment. In contrast, HSCGT rescued the atrophic muscularis propria of the small intestine in treated *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice 10 months after treatment. This suggests that the myogenic changes can be reversed when gene therapy is provided early in life. Finally, in **Chapter 6**: we further evaluated in depth the LV vectors integration profiles in gene modified human, rhesus or murine HSCs cultured *in vitro*, and *in vivo* after transplantation in disease models of SCID, Pompe, MNGIE and healthy mice. The integration patterns were similar in all species studied, and were not skewed based on the type of promoter (SF versus PGK, RAG1p, TCRβp, UCOE and γcPr), or transgene used ( *GFP* versus *Il2rg, Rag1*, *Rag* 2, *Gaa* and *Tymp*).

In conclusion, the results presented in this thesis demonstrate efficient and persistent correction of the biochemical phenotype in affected organs of *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice, brain neurological phenotypes (white matter edema and astrocytes morphology), and intestinal myopathy (atrophy of the muscularis propria), at relatively low LV-PGK-TP(co) vector copy number per cell and without obvious vector or disease related phenotoxicity or genotoxicity. Due to the lack of some of the major neurological or gastrointestinal clinical phenotypes in *Tymp*$^{-/-}$ *Upp1*$^{-/-}$ mice (such as motor dysfunction or cachexia) we should be cautious in translating our findings from mice to MNGIE patients. Nonetheless, it is recommended to perform more studies before clinical application, including evaluating HSCGT in more clinically relevant models that mimic human MNGIE disease (e.g. *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice fed with a diet to enforce the phenotypes), dosing of LV vector and transplanted gene modified cells, and more thorough assessment of vector biodistribution and safety studies addressing potential phenotoxicity and genotoxicity in depth.

# | SAMENVATTING (DUTCH SUMMARY)

Hematopoietische stamcelgentherapie (HSCGT) is een aantrekkelijke behandelingsoptie voor een breed scala van aandoeningen. Bij de eerste klinische studies naar X-linked ernstig gecombineerde immunodeficiëntie (SCID-X1) en adenosine deaminase (ADA)-SCID heeft men γ-RV vectoren met succes toegepast, maar bij SCID-X1 ontstonden hematologische maligniteiten als gevolg van vectorintegratie en transactivatie van nabije oncogenen. Daarom werd gezocht naar alternatieve virale vectorsystemen. Dit leidde uiteindelijk tot de ontwikkeling van de nieuwe, derde generatie zelf-inactiverende (SIN) lentivirale (LV) vectoren met verbeterde efficiëntie en goedaardige integratieprofielen.

Mitochondriële neurogastrointestinale encefalomyopathie (MNGIE) wordt momenteel behandeld met allogene hematopoietische stamceltransplantatie (allogene HSCT). Deze therapie leidt tot reversie van het biochemische fenotype, maar is geassocieerd met hoge sterftecijfers. Verder dienen matchted donor hematopoietische stam- en voorlopercellen (HSPCs) beschikbaar te zijn, en er is nog weinig bekend over de uitkomst op de lange termijn. LV-gemedieerde HSCGT maakt gebruik van autologe cellen, kan mogelijk toe met lagere pre-conditioneringsregimes en kan naar verwachting systemische ziekte op termijn corrigeren, zoals besproken in **Hoofdstuk 2**.

In **Hoofdstuk 3** optimaliseerden we de *ex vivo* kweekomstandigheden van LV-vector gemedieerde genoverdracht in hematopoietische stam en voorloper cellen (HSVC's). Het doel hiervan was om efficiënte transductie te bereiken binnen een korte incubatietijd, met een minimaal aantal geïntegreerde vectorkopieën en minimale cytokine toevoegingen. De resultaten geven aan dat het haalbaar is de genoverdracht te verbeteren door het verhogen van de dichtheid van muizen-, rhesus- of humane HSVC-cellen. Ook zagen we dat de overnacht transductie van muis- en humane HSVC's in stamcelmedium waaraan alleen trombopoëtine (TPO) was toegevoegd als cytokine, significant verbeterde transductie opleverde voor *in vivo* doeleinden. Toevoeging van relatief lage concentraties van de groeifactoren SCF, TPO, Flt3-L gaf de meest efficiënte genoverdracht (60% GFP-markering) met behoud van de mogelijkheid tot repopulatie van voorloper- en stamcellen. Alles bij elkaar genomen bieden deze bevindingen mogelijkheden om de beperkingen van de huidige genoverdrachtprotocollen te verbeteren door een verhoogde efficiëntie en minder veiligheidsrisico's m.b.t. insertionele mutagenese, met minimale *ex vivo* manipulatie gedurende een korte kweekperiode en de toegevoegde commerciële waarde van het gebruik van kleine hoeveelheden klinische lentivirale vectoren en cytokines.

In de **Hoofdstukken 4 en 5** lag de nadruk op de evaluatie van de efficiëntie en veiligheid van LV vector-gemedieerde hematopoietische stam en voorloper cel gentherapie (HSVGT) als een behandeling voor MNGIE in *Tymp* $^{-/-}$ *Upp1* $^{-/-}$ *muizen*. Hierbij werden therapeutisch toepasbare SIN LV-vectoren gebruikt met de fysiologische humane fosfoglyceraatkinase (hPGK) promotor om algemene expressie van oorspronkelijk- of codon-geoptimaliseerd humaan TYMP cDNA te bewerkstelligen (respectievelijk PGK-TP of PGK-TPco).

Voor evaluatie van de veiligheid werd de LV-SF-TPco gebruikt met de sterke milt-focusvormende viruspromotor waarbij een hoge TP-expressie wordt verkregen om de mogelijke genotoxiciteit te beoordelen. De resultaten in **Hoofdstuk 4** wezen op persisterende TP-activiteit en klaring van nucleosiden in de hersenen van behandelde *Tymp*$^{-/-}$ *Upp1*$^{-/-}$ muizen bij relatief lage aantallen vector-kopieën (PGK-TP(co): MOI 10, bereik, 0,2-3,6 VCN /cel). Dit resulteerde niet alleen in de correctie van de fenotypische veranderingen die te zien waren in de hersenastrocyten van *Tymp*$^{-/-}$ *Upp1*$^{-/-}$ muizen, maar ook in vermindering van oedeem in de witte stof, zoals aangetoond met hersen-MRI en immunohistochemie. Bij de lange termijn follow-up van de van behandelde HSCGT muizen zijn geen nadelige bijwerkingen van de procedure gezien: ***(i)*** Falen van donormateriaal of toxiciteit door de subletale bestraling van het gehele lichaam zijn niet waargenomen. ***(ii)*** De overlevingscurven van de muizen in de PGK-TP(co) behandelingsgroepen waren statistisch niet significant verschillend van de overlevingscurven van de controledieren. ***(iii)*** LV-gerelateerde insertiemutagenese of hematopoietische transformatie werden niet waargenomen na in totaal 22 maanden follow-up van de primaire en secundaire ontvangers van door therapeutische LV-vectoren (PGK-TP(co)) getransduceerde HSC's. ***(iv)*** LAM-PCR en sequencing lieten een polyclonaal integratiepatroon zien zonder bias naar integratie in de nabijheid van of in proto-oncogenen.

Gastrointestinale aandoeningen komen veel voor en zijn de belangrijkste doodsoorzaak bij MNGIE-patiënten. In **Hoofdstuk 5** hebben we onderzoek gedaan naar de pathologie van de dunne darm van MNGIE-patiënten en *Tymp*$^{-/-}$ *Upp1*$^{-/-}$ muizen en de effecten van respectievelijk allogene HSCT of gentherapie. Onze bevindingen bevestigden de al eerder gerapporteerde atrofie van de muscularis propria en afwezigheid van interstitiële cellen van Cajal bij MNGIE-patiënten. HSCT liet geen effect zien in de korte tijd tot enkele maanden na transplantatie. Dit kan de herhaaldelijk gemelde aanhoudende gastrointestinale klachten na de behandeling verklaren. Daarentegen herstelde HSCGT de atrofische muscularis propria van de dunne darm in behandelde *Tymp*$^{-/-}$ *Upp1*$^{-/-}$ muizen 10 maanden na de behandeling. Dit suggereert dat de myogene veranderingen omgekeerd kunnen worden wanneer gentherapie vroeg in het leven wordt gegeven. Tenslotte, **Hoofdstuk 6** betreft een diepgaandere evaluatie van de LV vector integratieprofielen in gen-gemodificeerde humane, rhesus of muizen HSVC's, gekweekt *in vitro* en *in vivo* na transplantatie in ziektemodellen van SCID, Pompe, MNGIE en in gezonde muizen. De integratiepatronen waren vergelijkbaar in alle onderzochte species en niet afhankelijk van het type promotor (SF versus PGK, RAG1p, TCRβp, UCOE en γcPr) of het gebruikte transgen (*GFP* versus *IL2RG*, *RAG-1*, *Rag 2*, *Gaa* en *Tymp*).

Concluderend blijkt uit de resultaten in dit proefschrift een effectieve en aanhoudende correctie van het biochemische fenotype in aangedane organen van *Tymp*$^{-/-}$ *Upp1*$^{-/-}$ muizen, hersen- fenotypes (witte stof oedeem en astrocyten morfologie) en intestinale myopathie (atrofie van de muscularis propria), bij een relatief aantal LV-PGK-TP(co) vector copien per cel en zonder duidelijke vector- of ziekte gerelateerde fenotoxiciteit of genotoxiciteit. Door het ontbreken van enkele

belangrijke neurologische of gastrointestinale klinische fenotypes in *Tymp*$^{-/-}$ *Upp1*$^{-/-}$ muizen (zoals motor dysfunctie of magerzucht) moeten we terughoudend zijn bij het vertalen van onze bevindingen bij de muizen naar MNGIE-patiënten. Het is aan te bevelen meer onderzoek te doen voordat dit klinisch kan worden toegepast. Zoals het evalueren van HSCGT in meer klinisch relevante modellen van humane MNGIE-muizen (bijvoorbeeld *Tymp*$^{-/-}$ *Upp1*$^{-/-}$ muizen die dieetvoeding krijgen om de fenotypen te forceren), de dosering van LV vector en getransplanteerde gen-gemodificeerde cellen, en verdere diepgaande evaluatie van de biodistributie van vectors alsmede grondiger veiligheidsstudies naar de mogelijke fenotoxiciteit en genotoxiciteit.

سلامة زيادة تركيز بروتين فوسفوريليز ثايميدين (TP)، ولتقييم مدى إمكانية وجود سمية ورائية. تشير النتائج الواردة في **الفصل الرابع** إلى وجود نشاط متواصل لبروتين TP واختفاء النيوكليوسيدات في دماغ الفئران *Tymp*$^{-/-}$*Upp1*$^{-/-}$ التي خضعت للمعالجة، وذلك بعدد نسخ منخفضة نسبياً (PGK-TP(co)): MOI 10، المدى: ٠,٢–٣,٦ VCN لكل خلية). هنا، قمنا بإظهار كيف أن هذا تسبب أيضاً في عكس التغيرات المظهرية التي حصلت في الخلايا النجمية (astrocytes) لدماغ الفئران من فئة *Tymp*$^{-/-}$*Upp1*$^{-/-}$ التي خضعت للمعالجة، كما أنها تسببت في تقليل وذمة المادة البيضاء (**white matter edema**) التي ظهرت في صورة الرنين المغناطيسي (**MRI**) التي أجريت للدماغ وتحاليل الكيمياء الهيستولوجية المناعية (**immunohistological analysis**). أظهرت المتابعة طويلة الأمد بعد تلقي العلاج الجيني للخلايا الجذعية الدموية (**HSCGT**) عدم وجود أي أعراض جانبية للعملية: *(أ)* لم يتم ملاحظة فشل الزراعة أو أي تسمم ناتج عن تعرض الجسم لأشعة سينية شبه قاتلة. *(ب)* لم تختلف منحنيات البقاء على قيد الحياة بشكل ملحوظ عند الفئران التي تمت معالجتها باستخدام PGK–TP(co) عن المدى الطبيعي. *(ج)* لم يتم ملاحظة أي طفرات إدراجية أو تغيرات في مكونات الدم مرتبطة بالفيروسات البطيئة (LV) بعد مدة وصلت إلى ٢٢ شهراً من المتابعة للمستقبلين الأوليين والثانويين للخلايا الجذعية التي تم تحويلها باستخدام الفايروس البطيء العلاجي PGK-TP(co). *(د)* أظهرت تحليلات LAM-PCR وتحليلات التسلسل وجود نمط اندماج متعدد النسائل من دون أي انحياز نحو حدوث اندماج بالقرب من أو في أي تجمع لطليعة الجين الورمي.

تعد مظاهر الألم في الجهاز الهضمي سائدة وتعد السبب الرئيسي للوفاة لدى المرضى المصابين بمرض منجي (**MNGIE**). في **الفصل الخامس** قمنا بفحص وتحليل الأمراض الظاهرة في الأمعاء الدقيقة لدى المرضى المصابين بمرض منجي (**MNGIE**) ولدى الفئران من فئة *Tymp*$^{-/-}$*Upp1*$^{-/-}$، إضافة إلى دراسة أثر زراعة نخاع العظم بالخلايا الجذعية (**allogeneic HSCT**) أو العلاج الجيني (**gene therapy**)، على التوالي. أكدت النتائج التي توصلنا إليها الادعاءات السابقة بحدوث ضمور في العضلة الخصوصة (**atrophy of the muscularis propria**) وعدم وجود أي خلايا خلالية من نوع كاجال (**Cajal**) لدى المصابين بمرض منجي (**MNGIE**). لم يتسبب العلاج بالخلايا الجذعية (**HSCT**) في حل هذه الخصائص المرضية خلال الفترة القصيرة التي تم دراستها بعد إجراء عملية الزراعة. يمكن لهذه النتيجة أن تفسر التغييرات الظاهرة في الجهاز الهضمي التي يتم الإبلاغ عنها بشكل متكرر لدى المرضى بعد تلقي العلاج. وعلى عكس ذلك، فإن العلاج الجيني للخلايا الجذعية الدموية (**HSCGT**) تسببت في تقليل ضمور العضلات المخصوصة في الأمعاء الدقيقة لدى الفئران من فئة *Tymp*$^{-/-}$*Upp1*$^{-/-}$ بعد ١٠ أشهر من تلقي العلاج. قد تشير هذه النتائج إلى أنن يمكن عكس التغيرات الناشئة من العضل في حال تم إعطاء العلاج الجيني في مرحلة مبكرة من الحياة. وأخيراً، في **الفصل السادس**، قمنا بإجراء تقييمات أكثر عمقاً لمكونات الدم مرتبطة بالفيروسات البطيئة (LV) لدى الكائنات التي طبقت عليها تعديلات جينية من البشر، أو القردة الصغيرة، أو الخلايا الجذعية المكونة للدم من الفئران مستنبتة في البيئة المخبرية وفي الوسط الحيوي بعد عملية الزراعة في الفئران المصابة بمرض العوز المناعي المكتسب الشديد (**SCID**)، داء اختزان الغلايكوجين النمط الثاني (**Pompe**)، ومرض منجي (**MNGIE**)، إضافة إلى فئران سليمة. كانت أنماط الاندماج متشابهة لدى جميع الأصناف التي تم دراستها، ولم يظهر أي اختلاف بناءاً على نوع المحفز المستخدم (SF, PGK, RAG1p, TCRβp, UCOE, γcPr)، أو الجين المستخدم (*GFP, Il2rg, Rag1, Rag 2, Gaa, Tymp*).

في الختام، فإن النتائج الواردة في هذه الرسالة تظهر تصحيح فعال ومستمر للنمط الظاهري البيوكيميائي في الأعضاء المصابة للفئران من فئة *Tymp*$^{-/-}$*Upp1*$^{-/-}$،

# (Arabic summary)| ملخص


يعد العلاج الجيني بالخلايا الجذعية (HSCGT) خيار علاجي مغر للعديد من الاضطرابات المرضية. أظهرت نتائج التجارب السريرية الأولية لمرضى داء نقص المناعة المشترك الشديد (SCID-X1) و (ADA-SCID) بأنها تتوافق بنجاح مع النواقل الفايروسية القهقرية من نوع جاما (γ-RV vectors)، إلا أن الأورام الدموية الخبيثة حصلت في حالة نقص المناعة المشترك الشديد (SCID-X1) بسبب اندماج الفايروسات القهقرية وعملية التفعيل النسخي للجينات المسرطنة المجاورة. وعليه، تم تركيز الجهود نحو إيجاد أنظمة فايروسية بديلة أدّت، في نهاية المطاف، إلى تطوير الجيل الثالث من الفايروسات البطيئة (LV) ذات التعطيل التلقائي (SIN) ذات الكفاءة العالية والتي تتمتع بفاعلية محسنة وبروفايلات إندماج حميدة.

في الوقت الحالي، يتم علاج مرض منجي (MNGIE) عن طريق زراعة نخاع العظم بالخلايا الجذعية (allogeneic HSCT). تنجح عملية زراعة نخاع العظم بالخلايا الجذعية بعكس النمط الظاهري البيوكيميائي. إلا أن عمليات زراعة نخاع العظم بالخلايا الجذعية ترتبط بنسب وفيات عالية، وهي تتطلب وجود متبرع تتطابق خلاياه الجذعية وخلاياه السلفية (HSPCs) مع المريض، كما أن المعرفة بالنتائج العلاجية طويلة الأمد محدودة. يتم خلال العلاج الجيني للخلايا الجذعية الدموية (HSCGT) باستخدام الفايروسات البطيئة استخدام الخلايا ذاتية المنشأ، وقد تحتاج هذه العلاجات إلى متطلبات وإجراءات أقل للمريض ما قبل زراعة نخاع العظم ومن المتوقع أن تعطي تصحيح مرضي طويل الأمل، كما هو موضح في **الفصل الثاني**.

في **الفصل الثالث**، قمنا بتهيئة بيئة خارجية (***ex vivo*** **culture conditions**) مثالية لعمليات نقل الجينات من خلال الفايروسات البطيئة إلى الخلايا الجذعية والسلفية المكونة للدم (HSPCs) لضمان كفاءة نقل الجينات خلال فترة تنبيت قصيرة، وباستخدام أقل عدد ممكن من النواقل الفايروسية، والحد الأدنى من إضافات السيتوكينات. تشير النتائج إلى فاعلية تحسين نقل الجينات من خلال زيادة كثافة الخلايا الجذعية و السلفية المكونة للدم المستهدفة المستخلصة من الفئران، أو القرد الصغير، أو الانسان. علاوة على ذلك، فإن عملية نقل الجينات التي تتم بين ليلة وضحاها للخلايا الجذعية والسلفية المكونة للدم لدى الفئران والإنسان في مستنبت الخلايا الجذعية مع إضافة ثرومبوبويتين (TPO) فقط تسببت في تحسين عملية نقل المادة الوراثية لأغراض الدراسة في البيئة الداخلية (***in vivo***). إن إضافة عوامل النمو SCF، و TPO، و Flt3-L بتركيزات منخفضة نسبياً تسببت في نقل الجينات بالكفاءة الأكثر(بنسبة تفوق الـ 60% على مؤشر GFP)، مع الحفاظ على قدرة إعادة إنتاج الخلايا الجذعية والخلايا السلفية. في الإجمال، تساعد هذه النتائج في فتح احتمالات مثيرة للاهتمام لتخطي القيود التي تنتج عن بروتوكولات نقل الجينات الحالية: كفاءة أعلى وتحسين في مدى سلامة التصحيح الجيني، إضافة إلى الحد الأدنى من التعديلات على البيئة الخارجية خلال فترة تنبيت قصيرة، والقيمة الاقتصادية المضافة التي نحصل عليها عند استخدام كميات صغيرة من الفايروسات البطيئة والسيتوكينات.

يتم التركيز في **الفصل الرابع والفصل الخامس** على تقييم مدى كفاءة ودرجة أمان الفايروسات البطيئة في العلاج الجيني للخلايا الجذعية الدموية (**LV vector-mediated HSCGT**) كعلاج لمرض منجي (MNGIE) عند تطبيقها على الفئران من فئة *Tymp*$^{-/-}$*Upp1*$^{-/-}$. تم استخدام الفايروسات البطيئة (LV) ذات التعطيل التلقائي (SIN)، وقابلة للتطبيق علاجياً، والتي تحتوي على محفز كيناز الفُسفوغليسَرات الفسيولوجي الموجود في الإنسان (hPGK promoter) من أجل التعبير عن human TYMP cDNA الأصلي (PGK-TP) أو المعدل (PGK–TPco). تم استخدام الفايروسات البطيئة LV–SF–TPco، والتي تحمل محفزقوي (SF) لتقييم مدى

والأنماط الظاهرية لخلايا الدماغ العصبية (وذمة المادة البيضاء والخلايا النجمية)، والاعتلال العضلي في الأمعاء (ضمور العضلة المخصوصة) وذلك من خلال عدد قليل نسبياً لنسخ عامل LV-PGK-TP(co) لكل خلية، من دون حصول أي تسمم جيني أو تغير مظهري ظاهر. وبسبب وجود نقص في عدد من أهم الأنماط الشكلية العصبية أو الخاصة بالجهاز الهضمي لدى الفئران من فئة *Tymp*$^{-/-}$*Upp1*$^{-/-}$ (مثل الاختلال في الوظائف الحركية أو الدنف (متلازمة الهزال))، فإنه يتوجب علينا أن نكون حذرين عند ترجمة النتائج التي توصلنا إليها عند دراسة الفئران وتطبيقها على المرضى المصابين بمرض منجي (**MNGIE**). بالرغم من ذلك، فإننا ننصح بإجراء دراسات إضافية قبل القيام بالتطبيق السريري، على أن تشمل تلك الدراسات تقييم مدى فاعلية العلاج الجيني للخلايا الجذعية الدموية (**HSCGT**) في بيئة سريرية أكثر ملائمة تحاكي جسد الإنسان المصاب بمرض منجي (**MNGIE**) (مثل فئران من فئة *Tymp*$^{-/-}$*Upp1*$^{-/-}$ خاضعة لنظام غذائي يحفز الأنماط الظاهرية)، وجرعة معامل الفايروسات البطيئة (**LV**) والخلايا المزروعة المعدلة جينياً، إضافة إلى إجراء تقييمات أكثر عمقاً للتوزيع البيولوجي للفيروسات، إضافة إلى دراسات أكثر عمقاً تتطرق لإمكانية حدوث تسمم جيني (**phenotoxicity**) أو تغير مظهري ظاهر (**genotoxicity**).

# | LIST OF TERMS AND ABBREVIATIONS

| | |
|---|---|
| **AAV** | adeno-associated virus |
| **ADA-SCID** | adenosine deaminase-severe combined immunodeficiency |
| **ANT-1** | adenine nucleotide translocase type 1 |
| **BBB** | blood brain barrier |
| **BFU-E** | burst forming unit-erythroid |
| **BM** | bone marrow |
| **CD** | cluster of differentiation |
| **cDNA** | complimentary DNA |
| **CFU-GM** | colony forming unit- granulocyte monocyte |
| **CGD** | chronic granulomatous disease |
| **CIPO** | chronic intestinal pseudo obstruction |
| **CIS** | common integration sites |
| **CNT** | concentrative nucleoside transporter |
| **CPEO** | chronic progressive external ophthalmoplegia |
| **cPPT** | central polypurine tract |
| **CRISPR/Cas** | clustered regularly interspaced short palindromic repeats/ CRISPR-associated |
| **dCTP** | Deoxycytidinetriphosphate |
| **dTTP** | deoxythymidine triphosphate |
| **DNA** | deoxyribonucleic acid |
| **dNTP** | deoxyribonucleoside triphosphates |
| **dThd** | thymidine |
| **dUrd** | deoxyuridine |
| **ECGF1** | platelet-derived endothelial cell growth factor |
| **EE-TP** | erythrocyte encapsulated TP |
| **ENT** | equilibrative nucleosides transport |
| **ERT** | enzyme replacement therapy |
| **FIX** | coagulation factor IX |
| **FACS** | fluorescence activated cell sorting |
| **Flt3-L** | FMS-like tyrosine kinase 3-ligand |
| **G-CSF** | granulocyte colony stimulating factor |
| **GFAP** | glial fibrillary acidic protein |
| **GFP** | green fluorescent protein |
| **γ-RV** | gammaretrovirus |
| **GVHD** | graft versus host disease |
| **Gy** | Gray (irradiation dose unit) |
| **H&E** | hematoxylin-Eosin |
| **HIV** | human immunodeficiency virus |
| **HLA** | human leukocyte antigen |
| **hPGK** | human phosphoglycerate kinase |
| **HSCs** | hematopoietic stem cells |
| **HSCT** | hematopoietic stem cell transplantation |
| **HSCGT** | hematopoietic stem cell gene therapy |
| **HSPCs** | hematopoietic stem and progenitor cells |
| **iPSCs** | induced pluripotent stem cells |
| **IRES** | internal ribosome entry site |
| **LAM-PCR** | linear amplification mediated PCR |
| **LHON** | Leber's hereditary optic neuropathy |
| **Lin-** | lineage negative |
| **LV** | lentivirus |
| **LVIS** | lentiviral integration sites |
| **MBP** | myelin basic protein |
| **MDS** | mitochondrial DNA depletion syndrome |
| **MNGIE** | mitochondrial neurogastrointestinal encephalomyopathy |
| **MOI** | multiplicity of infection |
| **mRNA** | messenger RNA |

| | |
|---|---|
| **mtDNA** | mitochondrial DNA |
| **NT** | nucleoside transporter |
| **OLT** | orthotopic liver transplantation |
| **PCR** | polymerase chain reaction |
| **PEO** | progressive external ophthalmoplegia |
| **PLP** | proteolipid protein |
| **PTAH** | phosphotungstic acid-hematoxylin |
| **qPCR** | quantitative PCR |
| **RAG -SCID** | recombination activating gene 1 and 2 severe combined immunodeficiency |
| **RNA** | ribonucleic acid |
| **RRE** | Rev response element |
| **SCF** | stem cell factor |
| **SCID- X1** | X-linked severe combined immunodeficiency |
| **SF** | spleen focus forming virus |
| **SIN-LV** | self-inactivating LV |
| **TALENs** | transcription activator-like effector nucleases |
| **TP** | thymidine phosphorylase enzyme |
| **TPO** | thrombopoietin |
| **TYMP** | thymidine phosphorylase gene |
| **TYMPco** | codon optimized TYMP gene |
| **UCB** | umbilical cord blood |
| **VCN** | vector copy number |
| **VRAC** | volume regulated anion channel |
| **WAS** | Wiskott-Aldrich Syndrome |
| **WPRE** | woodchuck hepatitis posttranscriptional regulatory element |
| **ZFNs** | zinc-finger nucleases |

# PHD PORTFOLIO

**Name PhD student:** Rana Yadak
**Erasmus MC Department:** Neurology
**Research School:** Molecular Medicine (MolMed)
**PhD period:** Sept. 2011- Dec. 2017
**Promoter:** Prof.dr. P.A.E. Sillevis Smitt
**Supervisors:** Dr. N.P. van Til and Dr. I.F.M. de Coo

| | Year | Workload (ECTS) |
|---|---|---|
| **1- PhD training** | | |
| **General courses** | | |
| Laboratory animal science | 2012 | 3 |
| Basic Human Genetics | 2013 | 0.5 |
| Basic Introduction Course on SPSS | 2013 | 1 |
| Research management for PhD students | 2014 | 1 |
| Research integrity | 2015 | 0.3 |
| Biomedical scientific English writing | 2016 | 2 |
| **Specific courses** | | |
| 5B irradiation protection course | 2015 | 1.4 |
| **Seminars and workshops** | | |
| Annual MolMed day (2x) | 2012-2013 | 0.6 |
| NCBI & other open source software | 2014 | 1 |
| Toxicology of gene-modified hematopoietic cells- Hannover Medical school | 2014 | 0.6 |
| Photoshop and Illustrator CS 6 workshop | 2014 | 0.3 |
| **Presentations** | | |
| NVGCT- Lunteren (3x) | 2014-2016 | 3 |
| NVKN- Alkmaar | 2014 | 1 |
| ESHG- Milan | 2014 | 1 |
| YMF and Euromit- Tampere | 2014 | 1 |
| CELL- PID- Ankara | 2014 | 1 |
| The XXII$^{nd}$ Annual ESGCT congress- The Hague | 2014 | 1 |
| ASGCT 18$^{th}$ annual meeting - New Orleans | 2015 | 1 |
| 11$^{th}$ EPNS- Vienna | 2015 | 1 |
| International MNGIE consortium meeting- Innsbruk | 2016 | 1 |
| **Posters** | | |
| Spierziektecongres- Veldhoven (2x) | 2014-2015 | 0.6 |
| Euromit- Tampere | 2014 | 0.3 |
| ICNC- Amsterdam | 2016 | 0.3 |
| **National conferences** | | |
| NVGCT- Lunteren (3x) | 2014-2016 | 1.7 |
| **International conferences** | | |
| PERSIST annual EU-project meeting – Leukerbad | 2012 | 1.1 |

**PhD Portfolio** (continued)

| | Year | Workload (ECTS) |
|---|---|---|
| CELL- PID annual EU-project meeting- Stresa (2x) | 2012-2013 | 2.3 |
| ESHG- Milan | 2014 | 1.1 |
| YMF and Euromit- Tampere | 2014 | 1.7 |
| CELL- PID- Ankara | 2014 | 0.6 |
| The XXII$^{nd}$ Annual ESGCT congress- The Hague | 2014 | 1.1 |
| ASGCT 18$^{th}$ annual meeting - New Orleans 11$^{th}$ | 2015 | 1.1 |
| EPNS- Vienna | 2015 | 1.1 |
| International MNGIE consortium meeting- Innsbruk | 2016 | 0.3 |
| ICNC- Amsterdam | 2016 | 0.3 |
| **2-Teaching** | | |
| Supervising a student during her master's thesis | 2013 | 38 |
| **3-Others** | | |
| **Research visits** | | |
| Dept. of Immunology, Weizmann Institute of Science (4 months) | 2012 | |
| Dept. of Pathology, VUMC- Amsterdam (3 months) | 2015,2017 | |
| Lab of Neuromuscular and Mitochondrial Disorders, Vall d'Hebron research Institute- Barcelona (3 weeks) | 2015 | |
| **Travel grants** | | |
| Tebu Bio researchers travel grant to attend ESHG in Milan | 2014 | |
| Meritorious abstract travel award to attend the ASGCT 18$^{th}$ annual meeting in New Orleans | 2015 | |
| **Volunteer activity** | | |
| The XXII$^{nd}$ Annual ESGCT congress-The Hague | 2014 | |

# | LIST OF PUBLICATIONS

**Yadak R**, Sillevis Smitt P, van Gisbergen MW, van Til NP, de Coo IF. Mitochondrial Neurogastrointestinal Encephalomyopathy Caused by Thymidine Phosphorylase Enzyme Deficiency: From Pathogenesis to Emerging Therapeutic Options. Front Cell Neurosci. 2017;11:31.

Van Gisbergen MW, Voets AM, Starmans MH, de Coo IF, **Yadak R**, Hoffmann RF, et al. How do changes in the mtDNA and mitochondrial dysfunction influence cancer and cancer therapy? Challenges, opportunities and models. Mutat Res Rev Mutat Res. 2015;764:16-30.

Huston MW, Riegman AR, **Yadak R**, van Helsdingen Y, de Boer H, van Til NP, et al. Pretransplant mobilization with granulocyte colony-stimulating factor improves B-cell reconstitution by lentiviral vector gene therapy in SCID-X1 mice. Hum Gene Ther. 2014;25(10):905-14.

Khurana S, Melacarne A, **Yadak R**, Schouteden S, Notelaers T, Pistoni M, et al. SMAD signaling regulates CXCL12 expression in the bone marrow niche, affecting homing and mobilization of hematopoietic progenitors. Stem Cells. 2014;32(11):3012-22.

**Yadak R**, Cabrera-Pérez R, Torres-Torronteras J, Bugiani M, Haeck J, Huston MW, et al. Preclinical efficacy and safety evaluation of hematopoietic stem cell gene therapy in a mouse model of MNGIE. *(Submitted).*

**Yadak R**, Boot M, van Til NP, Cazals-Hatem D, Finkenstedt A, Bogaerts E, et al. Transplantation, gene therapy and intestinal pathology in MNGIE patients and mice. *(Submitted).*

Van Til NP *, **Yadak R***, Buffa V, Huston MW, Aerts-Kaya F, van Helsdingen Y, et al. Development of a highly efficient lentiviral gene transfer protocol for hematopoietic stem cells. *(To be submitted).*
*The authors contributed equally

Huston MW, Horsman S, Nowrouzi A, van der Velden G, Varkouhi A, Li Y, **Yadak R**, et al. Hematopoietic stem cell lentiviral integration profiles are stable across multiple species, disease phenotypes and hematopoietic cell types. *(To be submitted).*

# CURRICULUM VITAE


| | |
|---|---|
| **Name** | Rana Mahmoud AbdulRaheem Yadak |
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**Positions**

| | |
|---|---|
| 2011-2016 | PhD position: Dept. of Hematology and Dept. of Neurology, Erasmus University Medical Center, Rotterdam, the Netherlands |

**Education**

| | |
|---|---|
| 2011-2017 | PhD candidate, Biomedical Sciences, Dept. of Hematology and Dept. of Neurology, Erasmus University Medical Center, Rotterdam, the Netherlands<br>Thesis: *'Lentiviral Hematopoietic Stem Cell Gene Therapy for MNGIE'*<br>Promotion date: 19 December 2017 |
| 2009-2011 | MSc, Biomedical Sciences (Cell and Gene Therapy), Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel, Brussels, Belgium,<br>Thesis*: 'Role of BMP signaling in regulating SDF-1α expression in murine bone marrow derived stromal cells'* |
| 2004-2008 | BSc in Medical Laboratory Sciences, Faculty of Science,<br>An-Najah National University, Nablus, Palestine |

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*Rana*

*P.S.* There's more to publishing scientific papers than good science or advancing our career as scientists. Behind every word and between every line lies the curiosity, dedication, and sacrifice of the PhD students who helped bring it to fruition. This journey, filled with both tears and laughter, is what makes for a rewarding experience in publishing scientific work.

The results presented in this PhD thesis demonstrate the efficient and persistent correction of the biochemical phenotype in affected organs of *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice, brain neurological phenotypes (white matter edema and astrocytes morphology), and intestinal myopathy (atrophy of the muscularis propria), at relatively low vector copy numbers and without obvious vector or disease-related phenotoxicity or genotoxicity. Due to the lack of some of the major neurological or gastrointestinal clinical phenotypes in *Tymp*$^{-/-}$*Upp1*$^{-/-}$ mice (such as motor dysfunction or cachexia), researchers should be cautious in translating our findings from mice to MNGIE patients. Nonetheless, it is recommended to perform more studies before clinical application, including evaluating HSCGT in more clinically-relevant models that mimic human MNGIE disease, the dosing of LV vector and transplanted gene modified cells, and a more thorough assessment of vector biodistribution and safety studies addressing potential phenotoxicity and genotoxicity.