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Advanced Therapies for Inborn Errors of Metabolism:

Development of innovative approaches to treat Pompe Disease and Crigler-Najjar Syndrome

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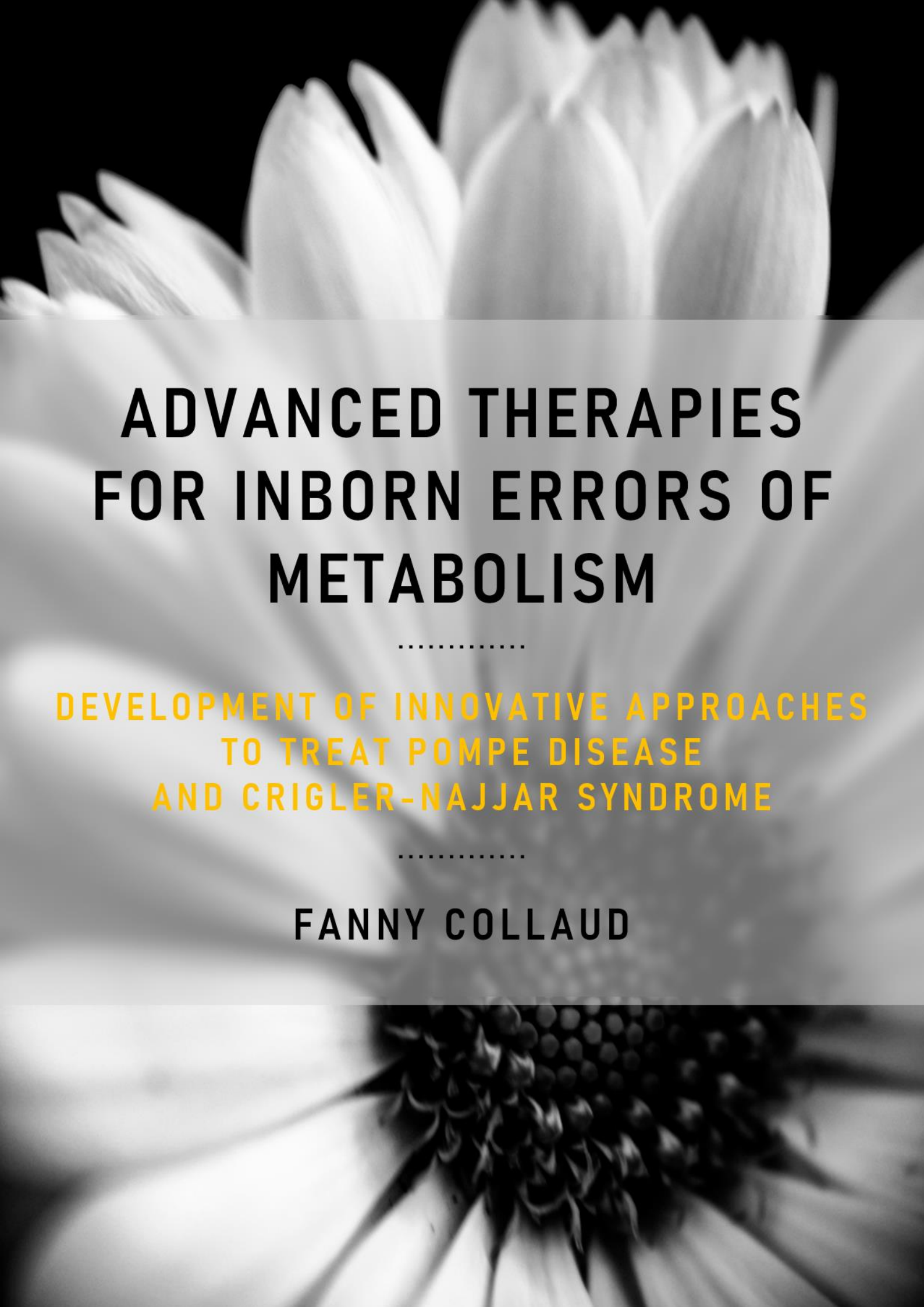

ADVANCED THERAPIES FOR INBORN ERRORS OF METABOLISM

DEVELOPMENT OF INNOVATIVE APPROACHES TO TREAT POMPE DISEASE AND CRIGLER-NAJJAR SYNDROME

FANNY COLLAUD

To my family,
so trustful and proud, since the very beginning

To my lab partners,
always supportive and motivated

To my mentors,
Federico, Giuseppe,
brilliant, passionate and inspiring scientists

To the CN community,
so that one day we turn off the light

To patients and their family,
our guiding force

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Table of content

List of abbreviations and acronyms

1-DNJ	1-Deoxynojirimycin
4MU	4-methylumbelliferone
AATD	alpha1-antitrypsin deficiency
AAV	adeno-associated virus
ABX	ambroxol
ADA	anti-drug antibody
AIP	acute and intermittent porphyria
AKU	alkaptonuria
ALS	amyotrophic lateral sclerosis
ASGCT	American Society of Gene and Cell Therapy
ASO	antisense oligonucleotides
BBB	blood-brain barrier
BCAA	branched-chain amino acids
BH4	tetrahydrobiopterin
BMT	bone marrow transplantation
CAR	chimeric antigen receptor
CB	conjugated bilirubin
ccALD	childhood cerebral form of X-linked adrenoleukodystrophy
CF	cystic fibrosis
CGD	chronic granulomatous disease
CIS	common integration sites
CLN2	ceroid lipofuscinosis type 2
CMV	cytomegalovirus
CN	Crigler-Najjar
CNS	central nervous system
CPEO	chronic progressive external ophthalmoplegia
CRIM	cross-reactive immune-material
CRISPR	clustered regularly interspaced short palindromic repeat
CRMs	cis regulatory modules
CSF	cerebrospinal fluid
CTL	cytotoxic T lymphocyte
DMD	Duchenne muscular dystrophy
DNJ-HCl	1-Deoxynojirimycin-hydrochloride
dsDNA	double-stranded DNA
eGFP	enhanced green florescent protein
EMA	European Medicine Agency
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
ERT	enzyme replacement therapy
ESCs	embryonic stem cells
FAODs	fatty acid oxidation disorders
FD	Fabry disease
FDA	Food and Drug Administration
FFPE	formalin-fixed paraffin-embedded
FH	familial hypercholesterolemia
GAA	acid alpha-glucosidase
GAMT	guanidinoacetate methyltransferase
GAN	giant axonal neuropathy
GCS	glucosylceramide synthase
GD	Gaucher’s disease

GILT	glycosylation-independent lysosomal targeting
GLP	good laboratory practice
GLUT1	glucose transporter type 1
GM-1	gangliosidosis type 1
GMP	good manufacturing practices
gRNA	guide RNA
GSDs	glycogen storage diseases
GSTs	glutathione-transferases
GT	gene therapy
GYS	glycogen synthase
GYS1	muscle-specific glycogen synthase
hAAT	human alpha 1-antitrypsin
HBB	human hemoglobin beta
HCC	hepatocellular carcinoma
HD	heterologous domains
HDR	homology-directed repair
HEK	human embryonic kidney
hFIX	human coagulation factor IX
HLCs	hepatocyte-like cells
HLP	hybrid liver-specific promoter
HRP	horseradish peroxidase
HSCT	hematopoietic stem cell transplantation
HSF1	heat-shock transcription factor 1
Hsp	heat-shock proteins
HSPCs	hematopoietic stem/progenitor cells
HSR	heat-shock response
HT-1	heridetary tyrosinaemia type 1
HTx	hepatocytes transplantation
IdeS	called Imlifidase
IEM	inborn error of metabolism
IOPD	infantile-onset Pompe disease
iPSCs	induced pluripotent stem cells
IS	integration sites
IS	immunosuppression
ISH	*in situ* hybridization
ITRs	inverted terminal repeats
KO	knock-out
KRAB	Krüppel-associated box
LAM-PCR	linear amplification mediated-PCR
LED	light-emitting diode
LGMDs	limb girdle muscular dystrophies
LGT	liver-directed gene transfer
LHON	Leber's hereditary optic neuropathy
LICLN	late infantile ceroid lipofuscinosis
LNP	lipid nanoparticles
LOPD	late-onset Pompe disease
LOQ	limit of quantification
LPL	lipoprotein lipase
LSDs	lysosomal storage disorders
LTx	liver transplantation
LV	lentivirus
M6P	mannose-6-phophate

MELAS	mitochondrial encephalopathy with lactic acidosis and stroke-like episodes
MePRDL	methylpredisolone
MIDD	maternally inherited deafness and diabetes
miRNA	microRNA
MLD	metachromatic leukodystrophy
MMA	methylmalonic academia
MOI	mMultiplicity of infection
MPS	mucopolysaccharidosis
mRNA	messenger RNA
MSC	mesenchymal stem
MSUD	maple syrup urine disease
mTOR	mechanistic mammalian target of rapamycin
mTORC1	mTOR complex 1
NAbs	neutralizing antibodies
NAC	N-acetylcysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NB-DNJ	N-butyl-deoxynojirimycin
NHEJ	non-homologous end joining
NHP	non human primate
NMD	nonsense-mediated messenger RNA decay
NPC	Niemann-Pick type C
nrLAM-PCR	non-restrictive variant of Linear Amplification Mediated-PCR
OAs	organic acidurias
OTC	ornithine transcarbamylase
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
PCs	pharmacological chaperones
PCT	pharmacological chaperone therapy
PD	Pompe disease
PEI	polyethyleneimine
PFIC	progressive familial intrahepatic cholestasis
PGK	phosphoglycerate kinase
PH1	primary hyperoxaluria type I
PKU	phenylketonuria
PMO	phosphorodiamidate morpholino oligonucleotide
PRs	proteostasis regulators
PT	phototherapy
PTC	premature termination codon
QCS	quality control systems
qPCR	quantitative real-time PCR
rAAVs	recombinant AAVs
rhGAA	recombinant human acid alpha-glucosidase
RNAi	RNA interference
RUSP	recommended uniform screening panel
SBMA	spinal-bulbar muscular atrophy
sc	self-complementary
shRNA	short hairpin RNA
SMA	spinal muscular atrophy
SRT	substrate reduction therapy
ss	single-stranded
TALENs	transcription activator-like effector nucleases
TB	total bilirubin

TFBS	transcription factor binding site
TFEB	transcription factor EB
Tregs	regulatory T cells
TRIDs	translational readthrough-inducing drugs
TTR	transthyretin
UCB	unconjugated bilirubin
UCDs	urea cycle defects
UGT	UDP glucuronosyltransferase
UGT1A1	UDP glucuronosyltransferase family 1 member A1
UPR	unfolded protein response
UTR	untranslated region
vg	vector genome
VGCN	vector genome copy number
WAS	Wiskott-Aldrich syndrome
WD	Wilson's disease
WMS	World Muscle Society
X-ALD	X-linked adrenoleukodystrophy
XLMTM	X-linked myotubular myopathy
X-SCID	X-linked severe combined immunodeficiency
ZFNs	zinc-finger nucleases

Chapter 1

General introduction and scope of the thesis

1. Overview of treatments for inborn errors of metabolism

1.1 Inborn errors of metabolism

Metabolism is the set of complex and incessant biochemical processes of matter and energy transformation that occur within the cells of living organisms. Organized into specific pathways of organic biosynthesis or degradation, it ensures vital activities such as respiration, heartbeat, brain nutrition, body temperature, digestion, etc.

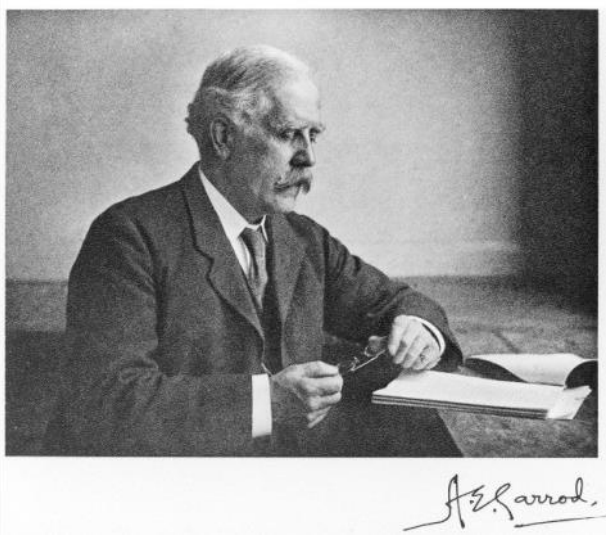

Figure 1.1 Portrait of Sir Archibald Garrod
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The concepts of "chemical individuality" and "inborn error of metabolism" (IEM) were introduced in the early 20th century by Sir Archibald Garrod (**Figure 1.1**), after his investigations to understand why the urine of a child was turned black. He "discovered" the Alkaptonuria (AKU) and its Mendelian inheritance.[1, 2] This landmark event led to the careful study of biochemistry in medicine and underlined the importance of working closely with the families to understand their pathologies. Two decades later, another important milestone in the developing field was the "discovery" of Phenylketonuria (PKU).[3] This disease was defined as a model in the study of inborn errors and all the work carried out on this pathology has contibuted to the development of revolutionary treatment concepts and newborn screening. Advances in chemistry and technology further favored IEMs identification, such as the first biochemical cycles description by Krebs or the advent of amino acids and organic acids analysis.[4-6]

Given the biochemical and technological knowledge of the past century, IEMs were initially described as monogenic disorders of the biosynthesis or breakdown of substances within a specific metabolic pathway, recognized by measurable abnormalities in biochemical tests and sometimes treatable by metabolic intervention. Since then, a recent definition suggests that IEMs should include more broadly any condition in which the impairment of a biochemical pathway is intrinsic to the pathophysiology of the disease.[7, 8]

While IEMs are individually very rare (with a frequency from 1:5,000 to 1:500,000), collectively they are very common, with an overall prevalence of about 1:1,000.[9, 10] Notably, it was established that IEMs result in up to 70% of all of the admissions to children's hospitals.[11] Since 1908 and the characterization of AKU as the first metabolic disease, over 1,000 different IEMs have been described worldwide and many others will continue to be characterized.[1, 8, 12-14]

IEMs can be classified in various way, based on their onset, predominant signs and symptoms, main organs or systems affected, acuity or chronicity of presentation.[15, 16] Among these disorders affecting metabolic pathways, three groups can be distinguished according to a common pathophysiology.

Diseases caused by intoxication constitute a first group. In this disorders, an accumulation of toxic compounds proximal to the metabolic defect, lead to a progressive or acute intoxication of the organism. This group includes various defects such as inborn errors of amino acid synthesis (serine, glutamine, and proline/ornithine) and amino acid catabolism (e.g. PKU, maple syrup urine disease (MSUD), homocystinuria, heridetary tyrosinaemia type 1 (HT-1), etc.), most organic acidurias (OAs) (e.g. methylmalonic, propionic, isovaleric, etc.), congenital urea cycle defects (UCDs), sugar intolerances (e.g. galactosemia, hereditary fructose intolerance), metal intoxication (Wilson, Menkes, hemochromatosis, and porphyrias), inborn errors of neurotransmitter synthesis and catabolism (monoamines, GABA, and glycine).

The diseases caused by energy deficiency, resulting from a defect in energy production or utilization within the liver, heart, muscle, brain, or other tissues, form a second group. They share common symptoms like hypoglycemia, hepatomegaly, hypotonia, failure to thrive, myopathy, cardiomyopathy, cardiac failure and brain involvement. In certain cases, they can also provoke developmental troubles such as dysmorphism, dysplasia, and malformations. This group of diseases can be further subdivided into mitochondrial and cytoplasmic energy defects. Mitochondrial disorders include fatty acid oxidation disorders (FAODs), ketone body defects, congenital lactic acidemias (defects of the Krebs cycle, pyruvate transporter, pyruvate carboxylase, pyruvate dehydrogenase), and mitochondrial respiratory-chain disorders. These pathologies are generally very severe and difficult to treat. Cytoplasmic energy defects encompass disorders of glycolysis, glycogen metabolism (such as glycogen storage diseases (GSDs)), gluconeogenesis, glucose transporter defects, errors of the pentose phosphate pathways, hyperinsulinism, and also creatine metabolism defects.

These first two groups are often referred to as small molecule diseases or intermediate metabolism abnormalities, that is to say involving the metabolic pathways of proteins, carbohydrates and lipids, their relationships and their modes of regulation. Importantly, these diseases are at high risk for acute decompensation. These "metabolic distresses" can occur at any age, in different forms, with a wide spectrum of clinical presentations.[17] Indeed, people with these conditions present intermittent intoxication episodes, corresponding often to increased metabolic stress events (e.g. specific food ingestion, fever or concomitant illness). Intoxication episodes may be acute (vomiting, coma, liver failure, thromboembolic complications, etc) or chronic (failure to thrive, developmental retardation, cardiomyopathy, etc.).

The third and last group are diseases caused by cellular organelles dysfunctions or other defects of complex molecules metabolism. Contrary to the previous groups, people affected by these pathologies present progressive but permanent symptoms, not related to intercurrent events. This group comprises lysosomal storage disorders (LSDs), peroxisomal disorders, intracellular trafficking and processing defects (e.g. alpha1-antitrypsin deficiency (AATD), carbohydrate-deficient glycoprotein syndrome), and inborn errors of cholesterol and bile acid synthesis. Belonging to this class of IEMs, two specific examples can be particularly highlighted here and will be widely discussed in this thesis: a specific LSD that is the GSD type II (GDS II), also called Pompe Disease (PD), and a bilirubin metabolism defect called Crigler-Najjar (CN) syndrome.

Although IEMs characterization is often complicated and unclear in view of the rarity of certain pathologies, prompt diagnosis is of the highest importance. Indeed, early detection and rapid correction of the metabolic impairment may be often lifesaving. Since the early 2000s, significant efforts have been made to ameliorate early diagnosis, and the implementation of tandem mass spectrometry in modern newborn screening programs

allowed for identification of many IEMs from a single blood spot analysis.[18-22] However, in many health systems, among inclusion criteria that authorize the introduction of a disease in an early detection program, availability of an accepted treatment is a key principle.[23] This poses difficulties in the context of newborn metabolic screening. Fortunately, the number of treatable IEMs continues to increase with time, and care continues to improve, thus many of them are now included in newborn screening programs in various countries. For example in United States, the Secretary of the Department of Health and Human Services developed guidelines and nominated 35 conditions to be included in a newborn screening program named the Recommended Uniform Screening Panel (RUSP).[24] Disorders registered on the RUSP can be identified within 24 to 48hours after birth (when the infant is generally clinically asymptomatic), with specific and sensitive tests allowing for immediate management of the sick child. Among IEMs, PD and Mucopolysaccharidosis type I (MPS I) were the most recent disorders included in the RUSP, respectively in 2013 and 2015. However, in France in particular, there is some delay in extending neonatal screening to larger number of IEMs, which could be possible regarding current technical possibilities, knowledge of the natural history of the diseases and the development of specific therapies. A collective thought, guided by ethics, must be held in the patients interest, offering them the opportunity for an early treatment.

Of note, some disorders can be missed by the neonatal screening or cannot be detected by fluids analysis, requiring more complicated investigations (e.g. biopsies or cell culture). Moreover, some milder forms of disorders can also appear later in life. Therefore, it is really important that clinicians discuss IEMs during the differential diagnosis. Nowadays, many conditions related to metabolism defects can be detected by several simple laboratory tests, and online tools are available to help clinicians.[25, 26] Further, as the enzymatic defect and the genes encoding the enzymes have been identified in many IEMs, the detection of impaired enzymatic activity and/or the genetic mutation characterization allow for a diagnosis.[12]

Early management of IEMs diseases is essential. While current supportive treatments are in place for some of them to prolong survival, improve quality of life or heal disease complications, effective therapies are still lacking for many of these disorders.[9] That is explained, in part, by the conceptual challenge that IEMs are loss-of-function defects, arising from a defective enzyme/cofactor, rendering pharmacologic rescue difficult. Nevertheless, undeniable progress has been made in understanding IEMs biochemical and molecular impairments. The genomics and metabolomics era has revolutionized the field, allowing rapid identification of metabolites and helping to understand the functioning of entire pathways and their interactions. Today, the pathogenesis can be explained by a deficiency of an essential enzyme, the activation of an abnormal metabolism, or by the circulation of toxic metabolites. Accordingly, several therapeutic options were developed to manage these conditions (**Figure 1.2**) and in 2013 around 83 treatments were referenced, which leads us to believe that they are even more numerous today.[27]

1.2 Current management

Traditionally, the management of IEMs aimed at the reduction of abnormal metabolites, consists in dietary modulation, based on nutrients restriction to prevent intoxication or nutrients supplementation to prevent secondary deficiencies. Other treatment options have become available, including strategies based on defective enzyme replacement therapy (ERT) by intravenous protein infusions, or even direct toxic metabolites elimination.[28-30]

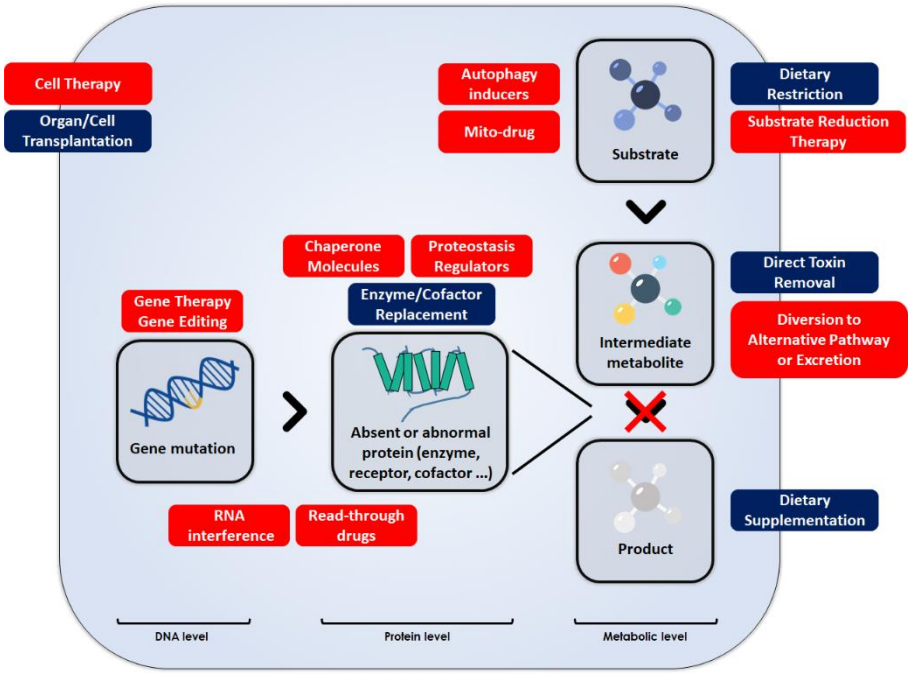


Figure 1.2 Therapeutic strategies for Inborn Errors of Metabolism (IEMs).
The basic principle of IEMs is illustrated. A genetic defect in enzyme or cofactor production leads to the toxic accumulation of a substrate, an intermediate metabolite or a decrease in production of product. All the different strategies discussed in this thesis are represented in the scheme, in blue (current treatments) and red (novel therapies) boxes. Adapted from Ginocchio et al, 2016 [29] and Gambello et al, 2018.[28]

Nutrition modifications

In the field of IEMs, dietary management is the keystone of therapy and sometimes the only effective intervention preventing disability or death.[31, 32] Indeed, many conditions are kept under control with a specific lifelong regimen, especially small molecule metabolic diseases (e.g. aminoacidopathies, OAs, UCDs, FAODs, or carbohydrate metabolism defects such as GSDs, etc.). This approach aims at restricting the intake of substrates or metabolites or at providing deficient products or alternative energy sources to bypass the defective pathway. The essential goal is to maintain normal growth and development of the sick child.

The more common dietary strategy is to restrict the intake of the nutrient whose degradation is not possible. To date, there are medical foods available for a wide range of conditions (e.g. galactose and lactose-free milks are available for patients affected by galactosemia; fructose, sucrose, and sorbitol-restricted diet for people suffering from hereditary fructose intolerance; zinc supplementation for people affected by Wilson's disease (WD), reducing the intestine copper intake; the protein substitutes and specific amino acid-free mixtures for PKU and other amino acid disorders; or diet low in saturated fat for Familial hypercholesterolemia (FH), etc.).[32-37]

In some IEMs, the pathogenesis is not only related to an accumulation of substrate, but also to the lack of the product of the affected pathway or to a defect in recycling of an essential factor. Product replacement therapy is used for example for patients with PKU, receiving tyrosine supplementation because of their inability to synthesize this amino acid.[38] Most frequently, the deficient product is glucose. Hypoglycemia can be provoked by reduced hepatic glucose production in case of hepatic GSDs, or excessive glucose consumption in case of FAODs. For these pathologies, frequent or even continuous feeding is often required. Some specific diet can extend fasting periods such as uncooked cornstarch.[39, 40] In Glucose

transporter type 1 (GLUT1) deficiency, a defect of glucose transport across the blood-brain barrier (BBB) leads to low cerebrospinal fluid (CSF) glucose concentration. Ketogenic diet can provide to these patients an alternative source of energy.[41] Concept of product supplementation is also applied for many other IEMs (e.g. in UCDs, where replacement of arginine or citrullin can reverse ammonia intoxication; in Tetrahydrobiopterin (BH4) deficiency, with neurotransmitter precursors L-dopa and 5-hydroxytryptophan administration ; in guanidinoacetate methyltransferase (GAMT) deficiency, where creatine can limit convulsive crises and slowdown neurological regression, etc.).[30, 42-45]

Catalysis of various enzymes implicated in IEMs pathogenicity are dependent on the binding with compounds that are not proteins, such as vitamins or minerals, which act as cofactors in metabolic processes. Supplying these cofactors can correct synthesis, transport or metabolic defects (e.g. vitamin B8 (biotin) supplementation in biotinidase deficiency, vitamin B1 (thiamin) in some variants of MSUD, vitamin B12 (cobalamin) supplementation for cobalamin disorders such as methylmalonic aciduria).[29, 30, 46]. They can also increase the enzyme residual activity by improving protein folding (e.g. BH4 enhances enzyme residual activity for PKU, and vitamin B2 (riboflavin) has positive effects on multiple acyl-CoA deficiency).[28, 47] This particular property will be addressed further. Importantly, the use of vitamin cocktail is common in neonates in critical conditions and with clinical suspicion of IEM. Unfortunately, although effective is some cases, not all patients respond to vitamins/coenzymes treatment.

The field of medical foods development is expanding continuously. In Europe, the market is mainly dominated by Nestlé Health Science (Vitaflo®), who elaborates formulas for many IEMs (**Figure 1.3**). While newer dietary therapies are explored to improve outcomes, dietary option is not an ideal solution for many reasons. First, the cost of these foods is elevated. For most of them, their flavors are not particularly palatable. It may seem trifling, but by the toddler and during childhood, taste becomes an important determinant of compliance. Diet schedule can affect quality of life and may not correlate with family demands.[48] Moreover, potential associated nutritional deficiencies can occur, requiring a careful oversight by a metabolic dietitian. Lastly, dietary modulation is often inadequate and fails to prevent long-term complications.

Figure 1.3 Example of medical foods
Niland Photography ©

Toxic Metabolite Disposal

Removal of abnormal accumulated toxic metabolites is an important strategy in the management of neonates presenting acute metabolic decompensation. In the case of certain small molecule metabolic diseases (such as aminoacidopathies, OAs and UCDs), branched-chain amino acids (BCAA: valine, leucine, and isoleucine) or ammonia are accumulated in the blood. These abnormal accumulations are very toxic to the brain and can lead to an acute encephalopathy, coma and irreversible cerebral damages. Their urgent removal is required, making a approach by diet modulation unappropriated. To achieve this, the toxin can be removed directly by hemodialysis, hemofiltration or peritoneal dialysis.[17] Scavenger

medications are nowadays available to divert the metabolic pathway or to provoke rapid excretion of the toxic metabolite in urine, and that will be further detailed in this manuscript.

Enzyme replacement therapy

Since many IEMs are caused by enzyme deficiency, various therapeutic strategies aimed at replacing the missing enzyme have been explored in the past 30 years. ERT, that consists in an intravenous administration of an exogenous functional enzyme, was firstly successfully used in Gaucher's disease (GD). Since then, it has provided treatments for previously untreatable IEMs, especially LSDs (e.g. Fabry disease (FD), PD, MPSs and lysosomal acid lipase deficiency).[28, 49] Indeed, the discovery of the lysosomal enzymes uptake pathway mediated by mannose-6-phophate (M6P) receptor, established the concept of cross-correction and the feasability of lysosomal enzyme supplementation[50-52] (**Figure 1.4**). ERT has greatly reduced the morbidity and mortality associated with LSDs, particularly in MPS I and PD. Thanks to the existence of this therapeutic option, these two diseases are now registered on the newborn screening panel in US, allowing an early management of pediatric patients. Newer ERT products are currently evaluated in the clinic, giving hope for the treatment of other IEMs (e.g. MPS IIIB, MPS VII, Niemann-Pick disease type B, Neuronal Ceroid Lipofuscinosis type 2 (CLN2)).[28]

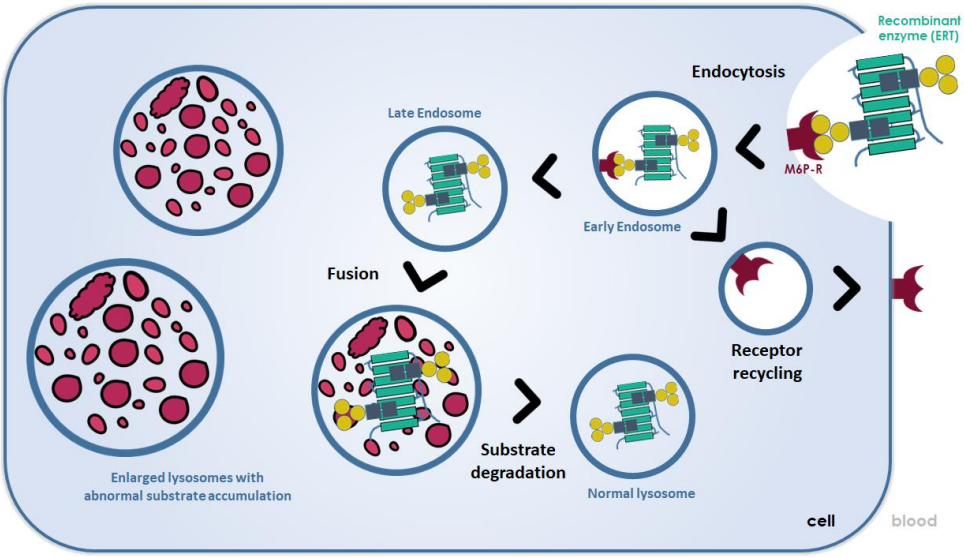


Figure 1.4 Lysosomal Enzyme Supplementation.
Enzyme replacement therapy (ERT) concept is based on the repeated intravenous infusion of human recombinant lysosomal enzymes. The enzyme binds to the mannose-6-phosphate receptor (M6P-R) present at the cell membrane, then it is internalized and delivered to lysosomes by endocytosis pathway. Once released into the lysosomes, it degrades the accumulated toxic substrate. Adapted from Parenti et al, 2012.[53]

Unfortunately, these treatments are not without drawbacks. Low half-life, stability and bioavailability of the recombinant enzymes limit efficiency of the treatment that requires frequent intravenous infusions to achieve efficacy, affecting patient quality of life. Available treatments are generally expensive and their real cost–effectiveness has been questioned: ERT can cost up to $200,000 per year, obviously representing a huge burden for the health systems.[54, 55] Another limitation is related to the absence of benefits of ERT for central nervous system (CNS) involvement, due to the inability of recombinant enzymes, systemically administered, to cross BBB.[49, 56, 57] It is also known that only a small fraction

of the recombinant enzyme can reach organs such as the bone cartilage or the eye for example, explaining why the correction of these tissues is limited even after long-term treatment.[58] Moreover, the possibility of severe and detrimental immune responses, often associated with the delivery of ERT, must be taken into account.[59-61]

Cell or organ transplantation

The scope of transplantation in IEMs is to transfer to the patient cells or tissue synthesizing the deficient enzyme to guarantee him a future as normal as possible, without dietary constraints and potential life risks. It can also replace an organ whose attack, secondary to the disease, dominates the symptoms, allowing to extand patient life.

Hematopoietic stem cell and bone marrow transplantation

Allogeneic hematopoietic stem cell transplantation (HSCT) and bone marrow transplantation (BMT) from healthy compatible donors have been established over the past 30 years as effective solutions for diverse IEMs affecting bone marrow function, such as hereditary immune deficiencies and thalassemia and have largely contributed to the improved survival and quality of life of children affected by LSDs (e.g. certain MPSs, Krabbe disease, GD, metachromatic leukodystrophy, X-linked adrenoleukodystrophy (X-ALD), fucosidosis and a-mannosidosis).[62, 63] After engraftment, healthy donor stem cells can colonize multiple tissues (including brain) of the affected patient and provide a constant source of enzyme that may be taken up by the enzyme-deficient cells via the mechanism of cross-correction. To date, while allogenic cells transplantation has benefited a limited number of conditions (e.g. MPS I, childhood cerebral form of X-ALD (ccALD)), this field is constantly in development (see the section *ex vivo gene therapy* detailed further) and the number of IEMs that can benefit from HSCT might expand in the future.

However, with this approach, it is known that therapeutic efficacy is closely dependent on an early stage intervention. Indeed, for IEMs with significant brain involvement, HSCT is effective only at pre-symptomatic stage or in patients with milder form of disease, the main clinical features being irreversible (e.g. neurodegeneration, skeletal deformation, etc.). Additionally, HSCT cannot guarantee that all organ have access to a proper enzyme restoration. This is particularly true for the brain, if the enzyme supplied to the periphery does not cross the BBB sufficiently to treat CNS pathology. Lastly, donor availability, graft failures, mixed chimerism and treatment-related morbidity and mortality represent serious limitations of HSCT.[29]

Liver transplantation

Since most metabolic processes happens in the liver, most IEMs affect, at least partly, this organ. Hepatologists encounter IEMs either in early infancy, when major symptoms typically manifest, or later when these patients are considered for liver transplantation (LTx). Their initial manifestations may often be similar to clinical features of chronic liver disease, presenting with acute liver failure syndrome (e.g. coagulopathy, conjugated jaundice, hepatocellular cytolysis, ascites, oedema), cholestatic jaundice (with failure to thrive), hypoglycemia and hepatosplenomegaly.[64] LTx is often the last option and until now it is considered the only curative treatment for a variety of these diseases.[65, 66] Currently, IEMs represent approximately 15–25% of indications for LTx in children.[67] IEMs amenable to LTx (**Table 1-1**) are not only disorders for which the main source of morbidity and mortality are hepatic injury. Indeed, LTx is also required in many liver-based genetic disorders

characterized by a structurally and functionally preserved liver, and for which extrahepatic complications are the main deleterious manifestations. In these conditions, the orthotopic LTx efficacy is remarkable with a 5-year survival rate of more than 90% for transplanted children.[68]

Table 1-1 Most common IEMs amenable to LTx

Disorders	Deficient enzyme	Tissue specificity	Liver features	Experiences in LTx for IEMs (%) in Europe and *(in Bergamo, Italy)**	
				in adults	in children
IEMs with hepatic expression and parenchymal damage					
Genetic cholestatsis				1.1 *(8)*	30 *(63)*
PFIC type 1	Familial intrahepatic cholestasis 1	Liver	Hepatomegaly, cholestasis, fibrosis, acute liver failure, cirrhosis, hepatocellular carcinoma		
PFIC type2	Bile salt export pump				
PFIC type 3	MultiDrug Resistant 3				
Alagille syndrome	Jagged1 or Neurogenic locus notch homolog protein 2	Liver, Heart			
Wilson's disease	Cooper-transport P-type ATPase	Liver, Kidney	Hepatomegaly, acute liver failure hepatitis, cirrhosis, hepatic coma	18.8 *(8)*	10 *(3)*
Hereditary hemochromatosis				13 *(32)*	nr
type 1	Hemochromatose modifier	all tissues, except Brain	hepatomegaly, cirrhosis, heptocellular carcinoma		
type 2a	Hemojuvein				
type 2b	Hepcidin				
type 3	Transferrin receptor 2				
type 4	Ferroportin				
Tyrosinemia type 1	Fumarylaceto-acetate hydrolase	Liver, Kidney	Hepatomegaly, acute liver failure, cirrhosis, hepatocellular carninoma	0.3	7 *(4)*
α-1-antitrypsin deficiency	Protease inhibitor	Liver, Plasma	Cirrhosis, hepatocelluar carcinoma	8.6 *(16)*	16
Argininosuccinic aciduria	Argininosuccinate lyase	Liver, Intestine	Hepatomegaly, fibrosis, cirrhosis in late-onset cases	nr	nr
Glycogen storage disease type 1	Glucose-6-phosphatase or Glucose-6-phosphate translocase	Liver, Kidney	Hepatomegaly, adenoma, hepatocellular carcinoma	1.1 *(8)*	4 *(5)*
IEMs with hepatic expression without parenchymal damage					
Urea cycle disorders				nr	nr *(6)*
NAGS deficiency	N-acetyl glutamate synthetase	Liver, Intestine	Near normal liver architecture in acute presentation		
CPS I defiency	Carbamoyl phosphate synthetase				
OTC deficiency	Ornithine transcarbamylase				
Argininemia	Argininase				
Citrullinemia	Argininosuccinate synthetase				
Crigler-Najjar syndrome	Uridine diphosphate glucuronosyltransferase	Liver	Normal liver architecture, traces of fibrosis in certain cases	0.4	4 *(5)*
Familial amyloid polyneuropathy	Transthyretin	Liver, retina, choroid plexus	Normal liver architecture	32.3	nr
Atypical heamolytic uremic syndrome -1	Complement factor H	Liver	Normal liver architecture	nr *(4)*	nr *(3)*
Primary hyperoxaluria type 1	Alanine-glyoxylate-aminotransferase	Liver	Normal liver architecture	3.4	7 *(5)*
Maple syrup urine disease	Branched-chain ketoacid dehydrogenase	all tissues	Normal liver architecture		
Acute intermittent porphyria	Porphobilinogen deaminase	Liver	Normal liver architecture	nr	nr
Homozygous familial hypercholesterolemia	Low-density lipoprotein (LDL) receptor, apolipoprotein B, pro-protein convertase subtilisin/kexin type 9 , or LDL receptor adapter protein 1	Liver, Heart	Normal liver architecture	nr	nr
IEMs with both hepatic and extrahepatic expression					
Organic acidurias				nr	nr *(6)*
Propionic acidemia	Propionyl CoA carboxylase	all organs	Normal liver architecture		
Methylmalonic acidemia	Methylmalonyl CoA mutase				
Cystic fibrosis	Cystic fibrosis transmembrane conductance regulator	Lungs, Pancreas, Liver, Kidneys, Intestine	Hepatic steatosis, focal biliary cirrhosis, rare cases of neonatal cholestasis	3.6 *(16)*	6
Erythropoietic protoporphyria	Mitochondrial enzyme ferrochelatase	Erythrocytes, Plasma, Liver	cholelithiasis, cholestasis, cirrhosis, acute liver failure	nr	nr
Gaucher disease	β-Glucocerebrosidase	Plasma, Liver, Kidney, Brain, Bone marrow	hepatomegaly, cirrhosis and acute liver failure in severe cases	nr	nr

*Adapted from Fagiuoli, et al.(2013)[66]; nr, not reported; *European liver transplant registry (1968-2010) and Bergamo (1998-2012) experiences in LTx for IEMs.*

However, the rationale for using LTx to replace an organ that is structurally normal (except for a specific pathway) raises questions about indications, timing, and priority. This is the reason why two alternative techniques, domino and auxiliary LTx, keeping the patient's liver intact, were attempted for these conditions and encouraging results have been reported for various IEMs (e.g. CN syndrome, UCDs).[69, 70]

In some diseases, where liver involvement is just one of the clinical manifestations, LTx is often only partially curative, and patients still require dietary management (e.g. methylmalonic academia (MMA), propionic acidemia). While LTx remains the standard therapy for several IEMs, this very invasive procedure, requiring extensive hospitalization

and rigorous adherence to a tapering regimen of medications, is still associated with a significant risk of complications and mortality. Following transplant, the risk of periodic rejection will always exist, requiring lifelong monitoring. The long-term consequences related notably to the life-long treatment with immunosuppressive therapy represent an additional issue.[68, 71] Moreover, the field of transplantation is faced with a worldwide shortage of organs.[72] In addition, selection for LTx is always a complex undertaking, and it is even more challenging in IEMs context, as risks of this complex surgico-medical intervention need to be carefully counterbalanced by the potential benefits of the metabolic correction. Indeed, while the list of potential IEMs amenable to treatment with LTx has increased, the evaluation of the risk-benefit ratio has become more complicated. Advances in understanding the pathogenesis of IEMs are essential to guide patient selection. This can only be achieved by a close collaboration between metabolic physicians and LTx team during any phase of the process.[64, 67]

Because of their prevalence and their severity, significant efforts have been made over the last years to develop novel and effective strategies and alternatives to LTx to cure IEMs.

1.3 Novel Therapeutic Approaches

While the first treatments developed for IEMs were largely aimed at reducing abnormal metabolites, new approaches focused more directly on the underlying metabolic defect correction. Promising therapies include small-molecule drugs, RNA targeting therapeutics, or cell and gene therapies. Of course, treatment of IEMs is a challenge, but the arsenal of drugs and therapeutic modalities will continue to grow in the coming years, improving with time their safety and efficacy, and giving hope to IEMs patients.

Small-molecule drugs

New compounds or drug repositioning

A new generation of techniques, such as informatics, Big Data and modeling methods, allowed for a better understanding of the pathophysiology of IEMs, development of new compounds, identification of biomarkers, etc.[73] Thanks to this growing knowledge, increasingly number of small-molecule drugs, which are able to stabilize, inhibit, or activate endogenous proteins and other molecules, or to act on a specific biochemical pathway, have been developed for IEMs treatment.[74] These advances have also highlighted the importance of current drugs repurposing, with the development of new drugs becoming more and more expensive. The modern high-throughput screening, presented as a fast track for the identification of novel therapies, has allowed the discovery of a large range of small-molecule drugs targeting a variety of molecular processes and will aid in providing new applications for existing drugs.[75] In fact, there are thousands of inexpensive and relatively safe drugs already approved for use in humans, that can be quickly repositioned to provide effective treatments for unsolved IEM. Here, few examples of new/existing compounds used for IEMs treatment will be discussed, more particularly pharmacological chaperones (PCs), proteostasis regulators (PRs), readthrough agents, substrate inhibitors, and autophagy inducers.

Pharmacological chaperones

Chaperone proteins are endogenous molecules that facilitate the folding and the trafficking of proteins and avoid their aggregation into the cell. Misfolded proteins, susceptible to

aggregate, are degraded by a quality control mechanism, the endoplasmic reticulum-associated degradation (ERAD) system.[76]

In several IEMs, genetic missense mutations result in protein misfolding or mistrafficking, leading to an early degradation of the concerned protein by the ERAD system and thus to a loss of an essential cellular function. In recent years, PCs have appeared as a promising therapeutic option for these IEMs. Described for the first time in 2000, these low molecular weight chemical molecules have the property to specifically bind to misfolded proteins, stabilizing their conformation and preventing their abnormal accumulation within the cell.[77, 78] Thereby they avoid an accelerated degradation by the ERAD and promote their trafficking (**Figure 1.5**).

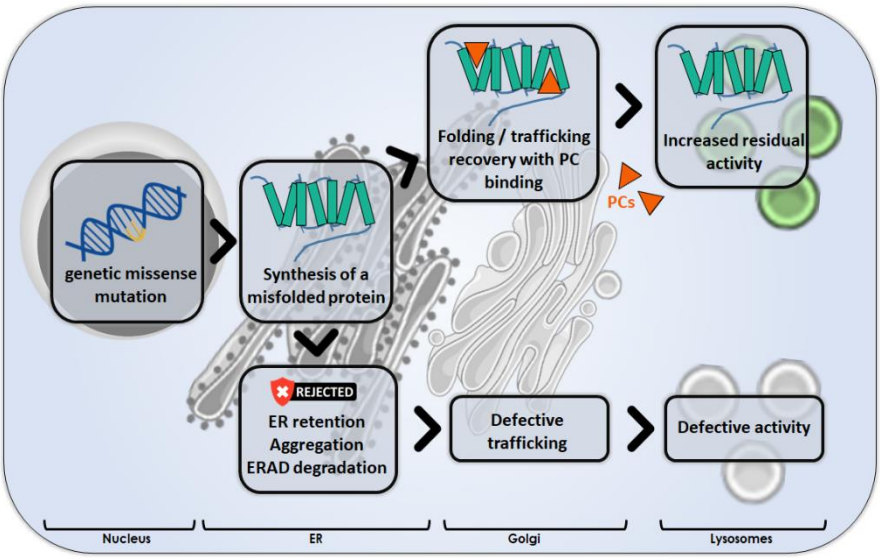


Figure 1.5 Mechanism of action of pharmacological chaperones applied for lysosomal storage disorders.
Mutated misfolded proteins are recognized by the quality control systems of the endoplasmic reticulum (ER) and are degraded, resulting in a defective enzyme activity. Pharmacological chaperones (PCs) can interact with the mutant protein, enhance its stability and restore its trafficking. As a result, the enzymatic activity of the mutant protein is partially rescued. ERAD, ER-associated degradation. Adapated from Parenti et al,2015.[79]

The PCs protein targets can have diverse subcellular localization (**Figure 1.6A**) and are in general proteins that transit in the endoplasmic reticulum (ER). They can be misfolded enzymes (in particular transferases) for which PCs can increase the residual activity, but they can be also transporters, receptors, regulators, or hormones.[80]

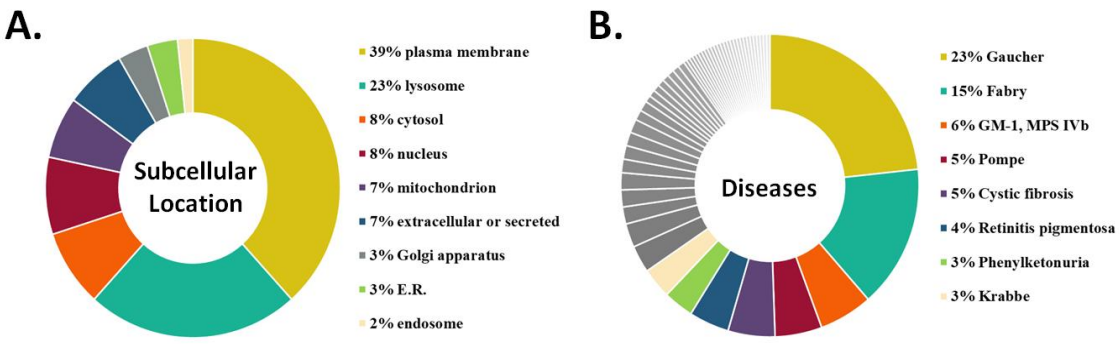


Figure 1.6 Pharmacological chaperones applications.
(A) Distribution of PCs protein target per cell localization. (B) Distribution of papers citing PCs per disease. The less represented diseases are in grey. Adapted from Liguori et al.,2020 [80].

Nowadays, PCs therapy (PCT) have been tested in more than 50 diseases and largely described in many reviews.[74, 80-85] The half of the literature covering the PCT subject concerns LSDs and more particularly GD, FD, Gangliosidosis type 1 (GM-1) and PD (**Figure 1.6B**).

Based on the molecular interactions that they have with the target protein, one can distinguish different kinds of PCs.[91] The vast majority of them are competitive inhibitors of the protein to be stabilized. This type of PCs has been largely studied for LSDs, promoting the misfolded enzyme trafficking to the lysosome, where the enzyme can degrade the accumulated toxic substrate. According on their operating mode, these PCs bind to their target proteins in the ER, at a neutral pH. This bond must be reversible so that, once arriving into the lysosome, the acidic pH enables the proteic complex dissociation. This dissociation is crucial to avoid any inhibition of the targeted protein. Hence, the balance between folding enhancement and enzyme inhibition of PCs is fragile.[86] Among the numerous examples of competitive inhibitors currently evaluated in IEMs, one can cite some compounds used in clinical trials for PD (NCTs 00688597, 04327973)[87-89], or other molecules such as migalastat (Galafold™, Amicus Therapeutics) for FD adult patients[90-93] or lumacaftor/ivacaftor (Orkambi®, Vertex Pharmaceuticals) for cystic fibrosis (CF) adult patients[94, 95], that were recently approved for marketing in both US and Europe.

Enzyme cofactors form a second group of PCs. As mentioned earlier in this manuscript, vitamins and minerals are commonly used in IEMs for their ability to stabilize proteins and act as chaperone of sort. Increased amounts of a natural cofactor aid in the stabilization of a misfolded enzyme and enhance the residual activity. The classical example of application of this approach is the BH4 supplementation for PKU management.[96] BH4 is the natural cofactor of phenylalanine hydroxylase, which is the defective enzyme involved in PKU.[97] In fact, in clinical practice, BH4-responsiveness is routinely tested in diagnosed PKU patients and this treatment is effective in almost half of the cases.[98] Because BH4 has also an important role in mitochondrial and energy metabolism regulation, this cofactor supplementation is under investigation in other IEMs such as hypercholesterolemia, diabetes mellitus and for various cardiovascular disorders.[99-103]

An emerging alternative group of PCs is represented by allosteric ligands. The advantage of these molecules is that they do not interact with the active site of the target protein. Thereby, they can rescue mutant enzymes that are not corrected by active-site PCs. Proof-of-principle studies highlight the efficacy of these molecules, such as for example, in induced pluripotent stem cell (iPSC)-derived macrophages and neurons in GD[104], or in a mouse model of PD[105]. A more relevant example is the Tafamidis meglumine (Fx-1006A, Vyndaqel®, Pfizer), an allosteric chaperone that has received market approval for Transthyretin (TTR)-related hereditary amyloidosis.[106] By selective binding of unoccupied enzyme sites, this molecule stabilizes TTR, slowing the progression of the disease. Allosteric PCs have also been used for the rescue of misfolded receptors, such as Frizzled-4 receptor.[107]

PCT presents many advantages. Firstly, PCs have, in general, a good safety profile. The oral administration of PCT reduces the impact on patient quality of life. Thanks to their small size, PCs exhibit a broad diffusion with the capacity to target most organs and tissues, including brain. PCs with high affinity for their target protein can be effective even at low doses.[79, 108] Their function also results in a near endogenous enzyme activity and lastly, they are non-immunogenic. PCT may also be combined with ERT, as is the case with PD.[87]

However, the use of PCT requires the presence of some residual endogenous enzyme and for this reason this therapeutic option is not applicable to all patients (e.g. when the protein

is absent because the gene is affected by a deletion, a stop, a splicing mutation, or a mutation occurring in the regulatory regions). Moreover, PCs are also mutation-dependent and not all disease-causing missense mutations are responsive to PCT. For some diseases, the percentage of responsive mutations is particularly low (for example PD).[109] Before attempting this kind of treatment, it is crucial to determine empirically if the disease of interest might be responsive to PCs. In this regard, *in vitro* experiments and some computational analysis can be useful to predict PCs efficacy on protein stability based on the mutation.[110-113]

Proteostasis regulators

Within the cells, a set of diverse biological pathways controls very carefully proteins biogenesis, folding, binding interactions, trafficking, and degradation. Collectively they are protein homeostasis or proteostasis pathways. The cell is thus equipped with protein quality control systems (QCS) in which chaperone molecules (such as heat-shock proteins (Hsp)), ubiquitin proteasome and autophagy play a crucial role. The cell responds to protein misfolding through different strategies depending on the compartment: the unfolded protein response (UPR), via activation of ATF6, PERK, and IRE1, in the ER and mitochondria, and the heat-shock response (HSR) in the nucleus and cytosol[114, 115] (**Figure 1.7**). These responses, modulated by different transcription factors, are made to manage small perturbations that occur within the cell and help misfolded proteins regain their correct conformation.[116] The maintenance of this delicate balance is essential to ensure vital functions, resistance to environmental stresses, and also to limit cellular perturbations after pathogen infections.

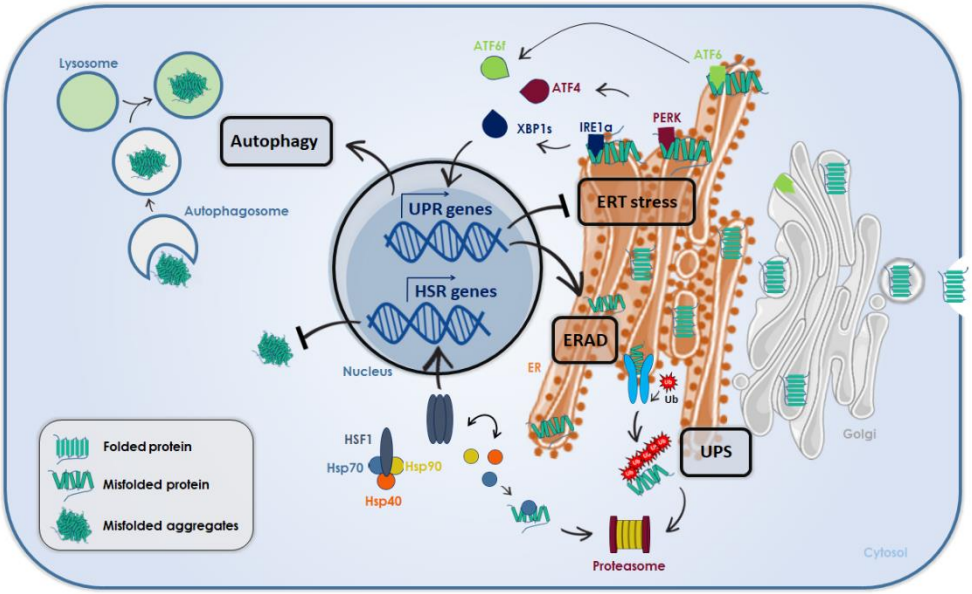


Figure 1.7 The proteostasis network and its quality control systems (QCS).
Schematic representation of the proteostasis actors including the unfolded protein response (UPR), the heat shock response (HSR), the endoplasmic reticulum (ER)-associated degradation (ERAD) pathway, the autophagy pathway, and the ubiquitin–proteasome system (UPS). All these mechanisms are connected to promote dynamic adaptation to protein-folding stress. The UPR is a complex signal transduction pathway in place to overcome the accumulation of misfolded proteins at the ER level or to trigger cell death in case of intense stress. The HSR is activated when accumulation of misfolded proteins is detected, notably by release, trimerization and translocation of heat shock factor-1 (HSF1). The autophagy pathway is in charge of the degradation of protein aggregates (or damaged organelles) by lysosomes and can be directly activated by the UPR. The ERAD is also modulated by the UPR and targets misfolded proteins from the ER to the cytosol, followed by their ubiquitination (Ub) and subsequent proteasomal degradation. Finally, some chaperones, such as heat-shock protein (Hsp)-70, can assist in protein degradation, leading misfolded proteins to UPS. Adapted from Mardones et al., 2015 [117].

IEMs associated with aggregation and degradation of misfolded proteins, leading to loss-of-function or aggregation-associated degenerative phenotypes, can interfere with the proteostasis maintenance. PRs are a class of small-molecule drugs able to influence and modulate this cellular mechanisms and restore proteostasis.[84, 118] There are several types of PRs acting in distinct cell compartments and able to mobilize molecular chaperones and/or to activate the protein QCS, mainly by acting on the heat-shock transcription factor 1 (HSF1), a key molecule in the coordination of HSR.

Non-steroid anti-inflammatory drugs, celastrol, Hsp90 inhibitors (such as geldanamycin), benzyl pyrazole derivative HSF1 activator and proteasome inhibitors (such as Bortezomib and MG-132) are all able to modulate HSR.[119-126] They act by either preventing the degradation of functional misfolded proteins or by reducing the abnormal level of toxic proteins. Although this therapeutic solution has been considered for several protein-folding diseases (e.g. GD and Niemann-Pick type C (NPC).[127, 128] The negative effects of a long-term inhibition of the proteasome activity has also been reported.[129]

The reduction of the ER calcium concentration and the consequent inhibition of the protein QCS at the ER level, small molecules such as curcumin or derivatives (e.g. BCM-95), thapsigargin, lacipidine, roscovitine and others, are under investigation with promising results in different IEMs (e.g. GD, CF, NPC, etc.).[130-135]

Autophagy inducers

Playing a role very similar to the small molecules just mentioned, autophagy inducers interfere, as their name indicates, in the autophagy signaling pathway thus allowing the clearance of potentially toxic proteins.

In normal conditions, autophagy is a self-digesting mechanism, which role is the elimination of malformed or non-functional intracellular components (including organelles) though lysosomes.[136] This process is strictly regulated by the mechanistic mammalian target of rapamycin (mTOR) through the mTOR complex 1 (mTORC1), which ensures a metabolic regulation of proteostasis, in response to intra- or extracellular factors such as cellular energy and nutrients levels, oxidative stress, ER stress, growth factors, etc[137-139] (**Figure 1.8**).

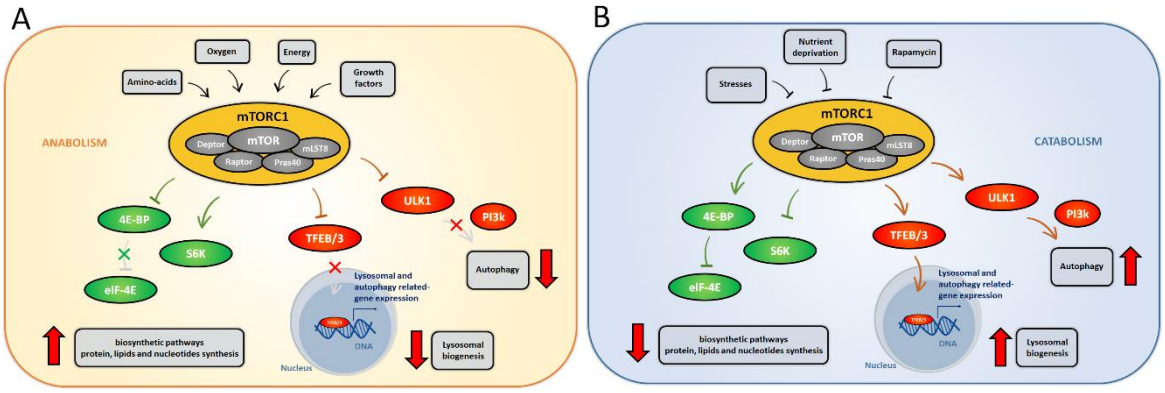


Figure 1.8 Schematic representation of the key signalling pathways in the regulation of autophagy via mTORC1.
The mechanistic target of rapamycin complex 1 (mTORC1) regulates a number of processes that affect the metabolic state of the cell. A range of physiological signals, such as growth factors, cellular energy and amino acids levels, affect the activation status of mTORC1, via interactions with the diverse components of the complex. (A). Under nutrient availability conditions, mTORC1 activation blocks autophagy by mediating phosphorylation-dependent inhibition of Unc-51 like autophagy activating kinase (ULK1) and the transcription factor EB/E3 (TFEB/3) on the lysosomal surface, while promoting cell growth by stimulating biosynthetic pathways, including the synthesis of proteins, lipids and nucleotides, via the inhibition of the translational repressor 4E-BP. (B).Under starvation, or other autophagy inducing conditions such as

ER stress or rapamycin, mTORC1 is inhibited and is dissociated from the ULK1 complex, relieving the inhibition of ULK1. ULK1 is then able to promote autophagy initiation and autophagosome maturation. In addition, mTORC1 inactivation leads to TFEB/3 dissociation from the lysosome and to its nuclear relocalization, where it can promote transcription of multiple genes regulating autophagy and lysosomal biogenesis. This catabolic metabolism allows the cell to maintain the critical level of energy and metabolites necessary to survive. Adapated from Rabanal et al., 2017 [140].

Many IEMs, specifically LSDs, affect lysosomal function or are associated with autophagy impairment.[141-143] For this reason, different strategies have been developed to modulate this pathway.[144, 145] Various approaches, mainly targeting TFEB or inhibiting the mTOR pathway and using autophagy inducers molecules such as rapamycin, genistein, cysteamine, hydroxypropyl-β-cyclodextrins, inositol-lowering compounds, or the antiepileptic drug carbamazepine, are currently evaluated for IEMs treatment (e.g. PD, nephropathic cystinosis, CF, NPC, Leigh syndrome).[146-155] Despite encouraging results, it is important to note that autophagy induction via mTOR signaling inhibition has been linked to some adverse effects, and the discovery of novel mTOR-independent modulators of autophagy is still an important medical need in IEMs.[156, 157]

Translational Readthrough-Inducing Drugs

Nonsense-mediated messenger RNA (mRNA) decay (NMD) is a cellular quality control pathway with the function to reduce errors in gene expression, by eliminating mRNA transcripts that contain premature termination codon (PTC). This mechanism, that protects the cell from the synthesis of unfunctional truncated proteins responsible of a large part of IEMs, represents a key strategy in these diseases.[74, 158] Translational read-through-inducing drugs (TRIDs) are small molecules that stabilize the ribosome and allow the translation machinery to overcome the PTC, elongate the nascent peptide chain, and consequently result in the synthesis of full-length protein, preventing the mRNA degradation by NMD.[159, 160] This molecules category, comprising aminoglycoside antibiotics (gentamycin, geneticin, etc.), PTC-124 (also called Ataluren) and NB84 (a designer aminoglycoside), has been widely investigated in a variety of pathologies, including IEMs (e.g. CF, MPS I, MMA, FD, etc.).[161-168] We can focus more specifically on PTC-124, which benefits since 2014 of a conditional European marketing authorization (under the brand Translarna®, PTC Therapeutics) for ambulatory patients with Duchenne muscular dystrophy (DMD). Despite the lack of a clearly demonstrated efficacy, this medication is presented as a potential therapeutic agent for diverse IEMs.[169-171] However, the more recent clinical data concerning patients with CF are not really enthousiatic.[172-175]

Some limitations are associated to the use of TRIDs. First, there are some evidences that only a small number of mutations generating PCT could benefit from these drugs, depending on the nucleotide context.[161, 176, 177] Then, the efficacy of protein restoration seems to be at best partial, limiting this approach to cases where only a small amount of protein is enough to improve the clinical phenotype. Another important limitation, is the potential immune response against the protein re-expressed by the drug in individuals that are naïve for that protein product.[159] All these reasons underline the importance of new molecules screening for more efficient therapies for diseases caused by PTC mutations.[178]

Substrate reduction therapy

Substrate reduction therapy (SRT) is an additional therapeutic modality for IEMs associated with a toxic buildup of substrate or where the accumulated metabolite is a complex molecule that is inappropriately stored within the cells (e.g. GSDs, LSDs).[179, 180] Instead of directly

repairing the defective enzyme, SRT aims to reduce the accumulation of the compound that cannot be fully metabolized (**Figure 1.9**).

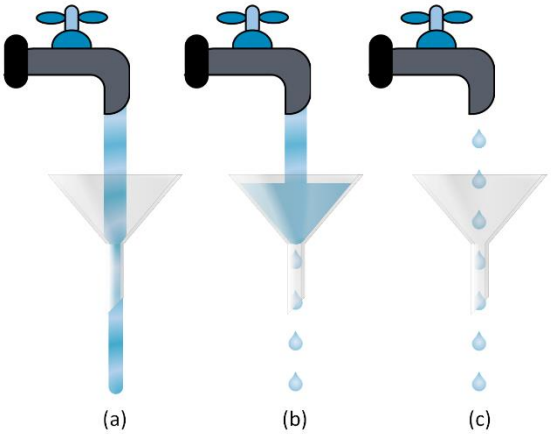


Figure 1.9 Visual representation of substrate reduction therapy
(a) in most individual cases, the substrate (water) can be degraded efficiently by adequate enzyme volume (hole in funnel). (b) in affected people, the amount of enzyme is insufficient to efficiently degrade the substrate and its accumulates. (c) in affected people treated by substrate synthesis reduction therapy, the amount of substrate is decreased to match the amount of residual enzyme to prevent accumulation. From © National Tay-Sachs and Allied Diseases, Inc. (https://www.ntsad.org)

With the current expansion of small-molecule drugs development, this therapeutic strategy has gained in interest, especially for IEMs that require the cross of the BBB to target the CNS.

In recent years, substrate inhibitors have become the basis of SRT for LSDs. For example, one of this drug, the glucose based iminosugar Miglustat (traded as Zavesca®, Actelion Pharmaceuticals), is used for inhibiting glucosylceramide synthase (GCS), which catalyzes glycosylceramide synthesis, precursor to the accumulating substances, in GD, and NPC.[181-185] The same substrate inhibitor can be used for a whole group of diseases sharing the same biosynthetic pathway. Similarly, Eliglustat (traded as Cerdelga®, Sanofi) a more selective GCS inhibitor is successfully used for the treatment of GD.[186-188] Nowadays, many other oral inhibitors are developed and evaluated for IEMs, with a focus on novel BBB-permeable inhibitors (e.g. the ceramide based Ibiglustat (Venglustat®, Sanofi Genzyme) in phase II/III trials for GM-2 (NCT04221451), GD (NCT02843035) and FD (NCT02228460); the galactose derivative Lucerastat (Idorsia Pharmaceuticals) for FD).

Moreover, other small molecules, are described to inactivate enzyme reactions that are upstream of the deficient enzyme to prevent accumulation of the toxic metabolites (e.g. nitisinone (NTBC, traded as Orfadin®, Sobi) for the treatment of HT-1 and AKU, genistein used to reduce accumulation of glycosaminoglycans in MPSs or phenylbutyrate (ACER-001, Acer Therapeutics) currently under clinical investigations in MSUD).[189-195]

These oral inhibitor drugs does not activate the immune system and overcome some of the limitations of the existing treatments. On the other hand, adverse effects were reported with this approach, as in HT-1 and AKU patients treated by nitisinone. Indeed, recent data suggest that this treatment is responsible of hypertyrosinaemia induction, which has been associated with cognitive impairment and potentially vision-threatening keratopathy.[196-198]

Derivation to alternative pathway

Representing alternative-pathway therapies for IEMs, some small-molecules drugs are used as scavenger medications to reduce toxin accumulation. The better described molecules are ammonia scavenger used in UCDs (e.g. carglumic acid (Carbaglu®, Recordati Rare Diseases Inc), the combination of sodium benzoate and sodium phenylacetate (Ammonul®,

Valeant Pharmaceuticals) or sodium phenylbutyrate). These oral drugs act by removing glycine and glutamate from plasma and by promoting an increased excretion of nitrogenated compounds, thereby reducing the accumulation of ammonia.[199-201] Other examples are the use of L-carnitine for the treatment of certain OAs and mitochondrial metabolism disorders and the administration of high doses of glycine for the treatment of isovaleric aciduria.[202, 203]

Mitochondria-targeted drug

Among the IEMs, mitochondrial disorders are quite common with a variety of diseases, both in term of clinical manifestation and genetic origin. Examples of these diseases are Leigh syndrom, Leber's hereditary optic neuropathy (LHON), Pearson syndrome, Friedreich ataxia, mitochondrial myopathies, chronic progressive external ophthalmoplegia (CPEO), mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), maternally inherited deafness and diabetes (MIDD), etc. Given the limited understanding of the precise nature of mitochondrias dysfunctions, very few therapies are, so far available for this group of diseases.

The role of mitochondria is to produce energy under the form of ATP. For that, the organelle is using oxidative phosphorylation (OXPHOS) complexes. Thanks to ATP synthesis, the mitochondria can act on metabolites oxidation by Krebs's cycle and β-oxidation of fatty acids. Furthermore, mitochondria also participate in many important cellular processes such as apoptosis and calcium homeostasis.

Most mitochondrial metabolic disorders are considered to be related to a deficit in ATP production, a reduced OXPHOS activity and a decreased oxidative capacity. For these reasons, various antioxidant small molecules are currently under investigation to modulate the oxidative stress (e.g. KH176, idebenone, EPI-743, coenzyme Q10, bezafibrate, vitamin E, MTP-131, RP103, and others) and they were recently reviewed.[204, 205] Further potential strategies, including mitochondria-targeted small molecules, are also being tested (e.g. rapamycin, resveratrol, nicotinamide riboside, inhibition of poly ADP ribose polymerase, NVP015, etc.).[204, 205]

Microbiome manipulation

We will finish this part by briefly evoking microbiome alteration, an alternative technique that is performed notably to reduce propionate production in methylmalonic or propionic acidemia, or trimethylamine in trimethylaminuria, by using antimicrobial agents.[206] In fact, little is known about the impacts of the metabolic default by itself or of some treatments taken by the affected patients (e.g. dietary modulation) on the microbiome. To date, some studies exploring IEMs and microbiome suggest a progressive onset of dysbiosis and a review of the literature concerning this specific topic was proposed recently.[207] This deregulation could induce or worsen metabolic abnormalities and thus contribute to liver and brain alteration. Consequently, engineered probiotics or specific antimicrobial agents have an interesting therapeutic potential and may constitute a simple intervention ameliorating the patients' life quality.

In summary, all the small-molecule drugs presented here have considerable advantages for treating certain IEMs, such as their potential to cross the BBB or their usual oral administration, avoiding potentially immunogenic exogenous proteins.[86] Moreover, they can be co-administered with other treatments or molecules to optimize their activity. Indeed, recent studies provided evidences of the synergistic effect of combination therapies, using together different compounds with different functions. To exemplify this approach, one can

refer to the works of Tatti and colleagues, where BCM-95, a small molecule, was associated to hydroxypropyl-β-cyclodextrin, an autophagy inducer, to enhance lysosomal function in the treatment of saposin C deficiency.[208] Similaraly, Williams and colleagues, associated curcumin, ibuprofen and miglustat to treat NPC.[209] However, some chemical drugs are molecules acting on specific mutations and cannot be prescribed for all patients, highlighting the limit of a personalized medicine. Today, given the high cost required to develop a new drug for a rare disease, efforts are focused in drug repositioning, which helps to not slow down treatments discovery for IEMs.

Hepatocyte cell-therapy

As previously evoked, state-of-the-art therapy for many IEMs is associated with significant shortcomings in terms of overall treatment efficacy and quality of life. For this reason, a search for novel treatments and even potentially permanent cures is highly desired.

As an alternative therapeutic option to the LTx, hepatocytes transplantation (HTx) is now investigated for some IEMs, more specifically in various inherited metabolic liver diseases (e.g. UCDs, PKU, GSD type I (GSD I), etc.).[210] This approach aims to provide organ support and promote liver regeneration. After enzymatic and mechanic dissociations, cells obtained from a donor liver are delivered via injection in the portal vein, a much less invasive procedure than LTx. Variable therapeutic benefit of HTx has been reported and even if in the majority of the cases it was only transient, encouraging clinical results were obtained.[211] The first attempts were made in patients with CN syndrome, where safety and efficacy were demonstrated, but the single cells infusion was not sufficient to completely treat the disease and the correction was transient.[212, 213] Promising results were also reported for one GSD I patient who still presented improved metabolic function 9 months after cell transplantation.[214]

While technically possible to realize in routine, the use of fresh or cryopreserved primary hepatocytes suspensions is limited by the supply in cells of good quality and organ availability. This major bottleneck, together with the risk of immune rejection, has slowed the widespread application of this liver cell therapy. Moreover, in a majority of inherited metabolic liver disorders, the liver architecture and the hepatocytes viability are normal, restricting the engraftment of transplanted cells. Indeed, with absence of a selective advantage, the normal-functioning hepatocytes have difficulties to replace host cells and provide enough correction to achieve clinical benefit. Of course, the minimal amount of cell engraftment required to achieve a therapeutic effect varies depending on the disease. For example, it was described for CN syndrome that a correction of only 5% of the liver mass was sufficient to provide phenotypic correction, while much higher percentages have to be achieved for other diseases, like FH or OAs. A summary of the clinical experiences of HTx was reviewed few years ago.[210, 211]

With the objective to improve the primary hepatocytes engraftment efficacy and to provide clinical correction, new standardized isolation, cryopreservation and storage protocols are now in development.[215] Some other alternative strategies are also being developed such as extrahepatic transplantation of cells or tissue engineering approaches.[216-218] New cell resources are also constantly being sought, such as progenitor and pluripotent liver stem cells (**Figure 1.10**). These cells, including embryonic stem cells (ESCs) and iPSCs, have the well-documented capacity to highly proliferate and differentiate into hepatocyte-like cells (HLCs).[219, 220] HLCs can also be derived from direct reprogramming protocols, such as from fibroblasts, with the important benefit to avoid immunosuppression.[221-223] Thanks to this

platform, patient-derived HLCs were developed as disease models for several IEMs (e.g. AATD, GSD Ia, FH) allowing to accelerate research for these pathologies.[224]

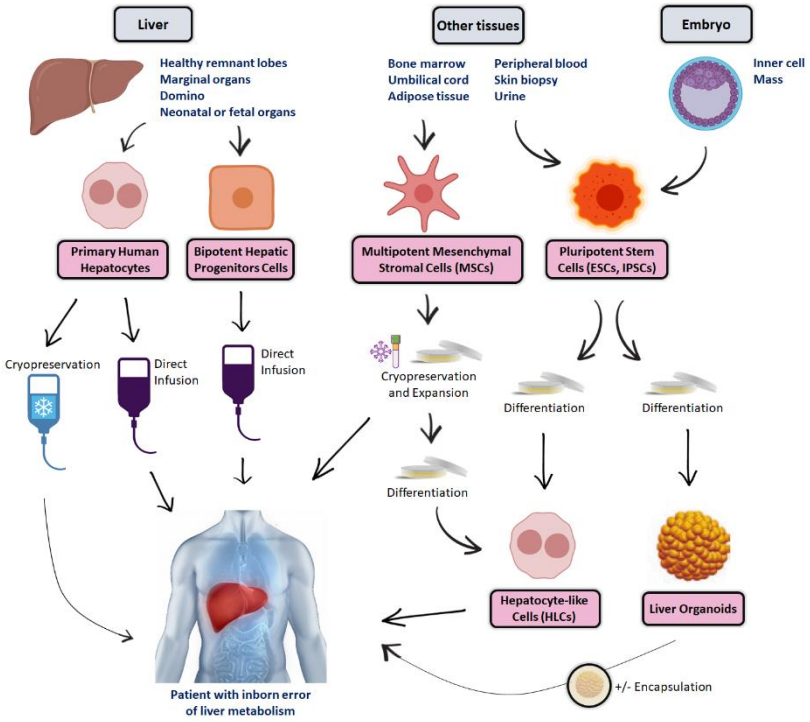


Figure 1.10 Cell sources for liver cell transplantation
Besides primary human hepatocytes (PHHs), several cell sources have been explored over the years as possible candidates for liver cell transplantation: bile duct-derived bipotent progenitor cells, multipotent mesenchymal stromal cells (MSCs), and hepatocyte-like cells or organoids derived from MSCs, embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Adapted from M. Pagenelli, 2019. [225]

The liver cell therapy is only at the beginning of its history and nowadays only few stem cell types are currently being clinically evaluated in IEMs. The stem cells used include bone marrow, adipose, and liver mesenchymal stem (MSC)/progenitor cells. Despite lot of technical progresses, uncomplete terminal differenciation of the cells represents a potential risk associated to teratoma formation after transplantation. Moreover, the engraftment efficacy and the clinical correction still need to be improved, as for example in the CN syndrome cell therapy trial that failed to demonstrate long-term efficacy and was suspended (NCT01345578).[226] However, the better understanding of the liver regeneration and differentiation mechanisms let imagine that the use of those cell types could be the near future solution if safety and engraftment efficacy can be further increased.

RNA-targeting therapeutics

Valuable knowledge has been gained in recent years about the central role of RNA and with it, the importance to develop strategies to modulate RNA.[227] Notably, we have only recently understood that genetic alterations can affect RNA functions and that RNA by itself may be responsible of pathologies through a mechanism called RNA-mediated toxicity.[228, 229] Examples of these RNA-mediated diseases are the disorders that present an expansion of repetitive nucleotide sequences (e.g. neuromuscular diseases myotonic dystrophies, spinocerebellar ataxia 8, Huntington's disease–like 2, Spinal-bulbar muscular atrophy (SBMA), fragile X-associated tremor ataxia syndrome, etc.) or those which have

deregulation of microRNA (miRNA) expression and alteration of miRNA binding sites.[230, 231] Given the RNA contribution to disease, the design of therapeutics that can directly target RNA, although challenging, represents a promising drug platform.

Antisense mechanisms, including RNA interference (RNAi), are perhaps the most direct therapeutic strategies to modulate mRNA function and are potential treatments not only for RNA-mediated diseases but also for several other disorders including a variety of IEMs.[232] This approach is based on molecules that bind specifically to RNA through Watson-Crick base pairing and affect the function of the targeted RNA by different mechanisms (**Figure 1.11**). Typically these molecules are small, single-stranded or double-stranded RNAs that contain in one strand a complementary sequence to the target mRNA. They can mimic natural sequences, such as existing miRNA, or be designed *in silico*. They can be delivered as synthetic oligonucleotides or as DNA templates inducing RNAi or other mechanism of action after transcription in the target cells. They can also be modified by addition of specific ligands (for hepatocytes targeting for example) allowing high efficiency targeting after systemic administration.[233]

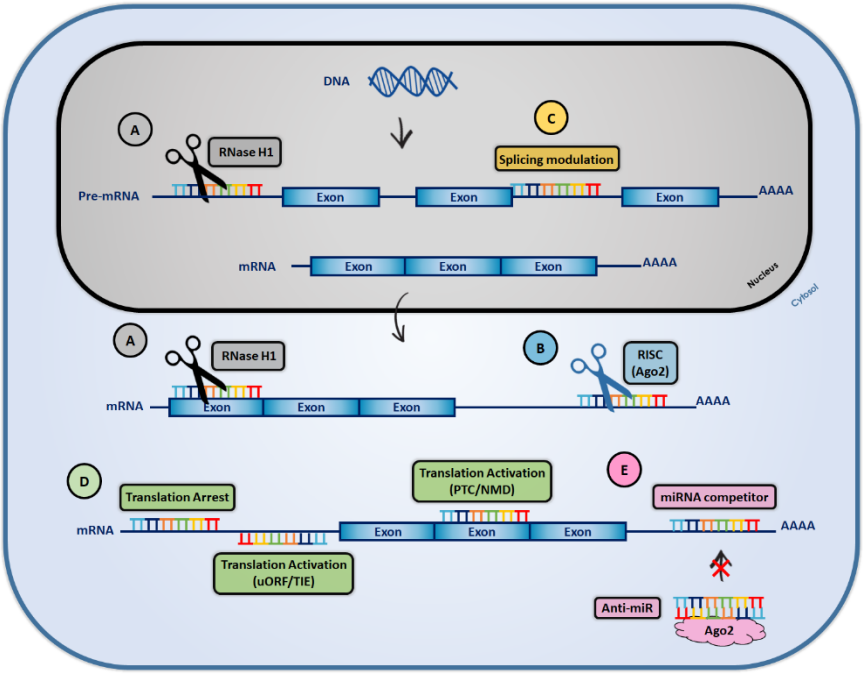


Figure 1.11 Antisense mechanisms commonly used to modulate gene expression
Antisense oligonucleotides can modulate gene expression through two different mechanisms based on post-hybridization events: enzymatic RNA degradation and occupancy-only mechanisms. Enzymatic RNA degradation includes (A) DNA-like antisense oligonucleotides that trigger complementary RNA cleavage by RNase H1 and (B) siRNA-mediated reduction of complementary RNA via the Ago2 RISC pathway, whereas other mechanisms such as ribozymes have also been explored. Occupancy-only mechanisms utilize antisense oligonucleotides to base pair with target RNAs without triggering RNA degradation. These include but are not limited to: (C) splicing modulation using non-DNA like antisense oligonucleotides to base pair with sequence elements in pre-mRNA to inhibit or enhance the utilization of splice sites; (D) translation modulation using non-DNA-like antisense oligonucleotides to base pair with mRNA, either to inhibit translation (steric blocking) or to activate translation through binding to inhibitory elements such as upstream open reading frames (uORF) or other translationinhibitory elements (TIEs); and (E) miRNA modulation either by base pairing with miRNA to inhibit the function of the miRNA or by base-pairing with miRNA-binding sites of a particular mRNA to eliminate the effect of a particular miRNA. Adapted from Crooke at al., 2018.[234]

So far, the antisense mechanism platform has been used for several applications in the clinic and constant improvements are made to optimize its safety, potency and delivery. A very rich literature is available on this topic and these tools were widely reviewed.[234-241]

Exogenously delivered mRNA therapy is emerging as a new therapeutic modality with the potential to treat myriad disorders, including loss-of-function IEMs. mRNA-based treatments can enter the affected cells and provide them the capacity to produce a given protein. Multiple mRNA-based therapeutics are now in clinical testing for prophylactic vaccines (e.g. Influenza, Zika or Corona viruses)[242-245] or cancer immunotherapies (e.g. myeloma, leukemia and glioblastoma, etc.)[246] and pharmaceutical industries invest a lot for the development of this new class of biologics.

Recently, significant progress has been made in the design of these mRNA to increase potency while reducing immunogenic profile. However, the widespread use of mRNA drugs requires the development of safe and effective drug delivery vehicles, which is the base of an efficient mRNA-based gene therapy. In the last years, the use of liver-targeting lipid nanoparticles (LNP) as vehicle, has proven safety and efficacy after intravenous administration in a variety of preclinical mouse models of IEMs (e.g. MMA, primary hyperoxaluria type I (PH1), GSD Ia, citrine deficiency, acute and intermittent porphyria (AIP), MSUD, arginase deficiency, ornithine transcarbamylase (OTC) deficiency, progressive familial intrahepatic cholestasis (PFIC), etc.).[247-256] Currently, Moderna Therapeutics is evaluating an investigational mRNA medicine (mRNA-3704) for MMA in a First-in-Human phase I/II study (NCT03810690). Usually mRNA-LNP administration is only effective for a maximum of 2-3 weeks, thus requiring repeated injections for a sustained correction, but these encouraging results support further investigations to propose mRNA therapeutics as possible treatments for IEMs.

Gene therapy

Twenty-two years: this is the time it took to go from the first human gene therapy trial to an official drug approved. To better understand this long journey, it is necessary to go back to the 1950s. At that time, scientists were already evoking the idea of modifying the human genome to correct diseases. However, the technical possibilities and knowledge were still too limited to move from the idea to a concrete application. In the following decades, with the development of biotechnologies, it finally became possible to manipulate DNA. Scientists learned and are learning more about the function of genes and their roles in diseases. During these years new systems for transferring therapeutic genes into cells (the "vectors") were discovered, in particular by relying on the use of viruses. All of this progresses, in 1989, enables Dr. Rosenberg at the National Cancer Institute to start a first trial of gene therapy in humans, based on the injection of genetically modified T cells in cancer patients.[257] The beginning of a great adventure.

Gene therapy became a growing hope for the scientific community and for patients. Unfortunately despite medicine progresses and breakthrough technologies, after the early promises and encouraging clinical outcomes, gene therapy has aroused caution and skepticism, due to repeated failures and the occurrence of serious adverse events in the 90s. Indeed, the fulminant systemic inflammatory response syndrome resulting in the death of an OTC–deficient patient, after first-generation adenoviral vector-based gene transfer in 1999, stopped the collective enthusiasm.[258] Moreover, later clinical trials, performed with gamma-retroviral vectors, although effective, reported the development of leukemia and myelodysplasia in patients with X-linked severe combined immunodeficiency (X-SCID), Wiskott-Aldrich syndrome (WAS), and chronic granulomatous disease (CGD).[259-264] Despite these very serious adverse events, these trials using gamma-retroviral vectors clearly demonstrated the potential of the gene therapy and resulted in the cure of some of the treated

patients. Since then, the scientists have learned the consequences of these setbacks and research efforts were focused on the development of more effective and safer protocols for the management of different diseases. As declared recently in a major medical journal "gene therapy comes of age".[265] In the last decade, the gene therapy field experienced a revival, thanks to a better understanding of the complexities of the vector–host interactions and to innovations in vector design. This resulted in several examples of clinical success, such as for congenital blindness[266-268], Hemophilia B[269-272], beta-thalassemia[273], WAS[274], XL-ALD and Metachromatic leukodystrophy (MLD).[275, 276] Although initially developed for inherited genetic disorders treatment, gene transfer technologies have been applied to a variety of diseases including cancers, cardiovascular diseases, infectious or autoimmune disorders. The success of treatment of blood cancers using chimeric antigen receptor (CAR)-T cells is considered one of the major successes of the gene therapy platform.[277] Today, gene therapy research is a growing and increasingly robust therapeutic approach. Indeed, 11 gene-therapy-based drugs are currently available in Europe, the USA and China (**Figure 1.12**) and according to the Food and Drug Administration (FDA) there are expected to be 40 by 2022.[278]

Figure 1.12 The approved gene therapy drugs

The next section will be devoted to gene therapy strategies developed so far for IEMs, with a specific focus on liver gene transfer.

2. Gene therapy for inborn errors of metabolism

2.1 Gene therapy strategies and vectors

Gene therapy involves introducing new genetic material (DNA or RNA) into cells to compensate the genetic default and consequently the deficient function. There are many gene therapy approaches, and initially, they were designed to replace a defective gene in case of monogenic disease (i.e. linked to the dysfunction of a single gene). But over the past two decades, the rapid evolution of knowledge and technology has made it possible to multiply possible strategies and broaden their use to include many indications, including certain cancers.

The various gene therapy strategies could be classified in five main categories (**Figure 1.13**) : i) gene replacement, the addition of a normal copy of the mutated gene; ii) gene supply, which is the overexpression of a gene that is not impaired which can prevent or smow down disease progression; iii) gene deletion or repair, known as genome editing, through targeted genetic modifications with sequence-specific molecular scissors; iv) targeted inhibition of gene expression, to avoid the deleterious consequences of mutated proteins exerting a toxic gain of function (see also above the **RNA-targeted therapeutics** section); and v) expression of a toxic protein by genetically-modified viruses or cells, typically used to kill cancer cells.

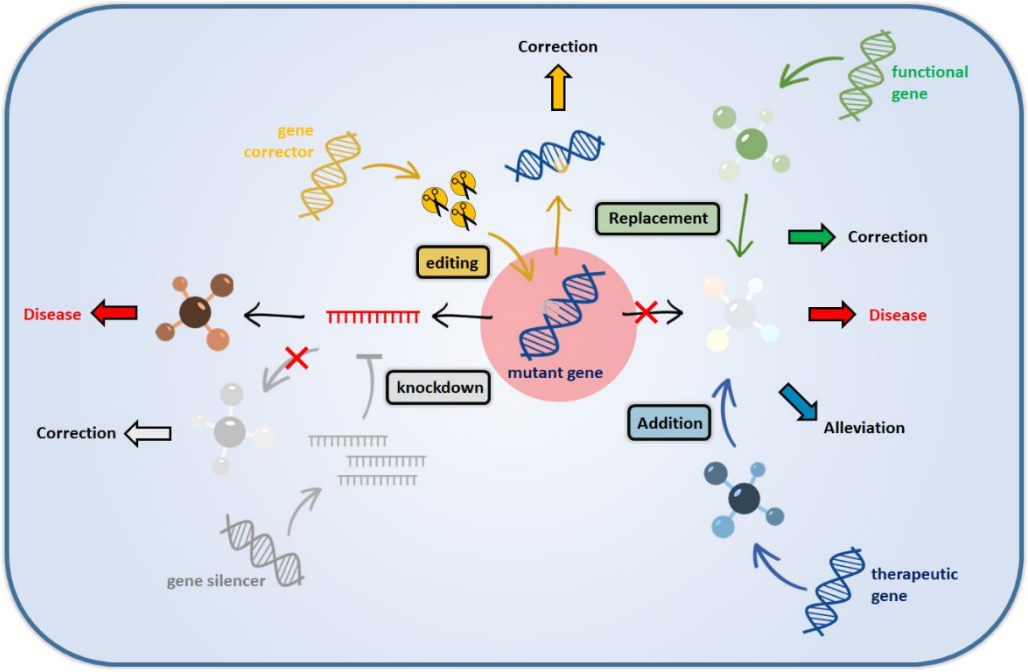


Figure 1.13 Gene therapy strategies.
A gene mutation reduces or abrogates protein synthesis (red cross) and leads to disease. Gene replacement corrects the disease by providing a functional copy of the gene (green helix) and normal proteins (green molecule). Gene addition alleviates the disease by supplementing therapeutic genes (blue helix) that target a specific aspect of the disease mechanism. In some cases, a gene mutation leads to the production of toxic protein aggregate (red molecule) that causes a disease. Gene knockdown utilizes small RNAs (grey combs) to inhibit the aberrant mRNA (red comb), thus preventing the synthesis of toxic protein aggregate and correcting the disease. Finally, gene editing by chimeric nucleases (yellow scissors) is a versatile approach to make a targeted change (grey dot) from a disease-promoting sequence to a disease-preventing sequence (yellow dot). This strategy can be used both to knock out or to knock down a gene. Adapted from Wang et al., 2014[279]

In principle, each of these strategies would not only ameliorate disease symptoms, but would also provide a definitive cure. Two main approaches are being pursued to correct genetic defects. *In vivo* gene therapy aims at the targeting of the gene directly to the cells of the organism. *Ex vivo* requires to genetically modify the patient's cells in the lab before they are re-injected into the patient. To date, several IEMs have been the objective of gene therapynvestigations in both *ex vivo* and *in vivo* approaches and more than 2,600 gene therapy clinical trials were completed, ongoing, or approved worldwide since the first use in 1989.[257] This chapter does not comprehensively depict the wide range of gene therapy approaches developed so far, but focuses on representative examples that have reached the clinic in patients with IEMs, and recently reviewed.[280-283]

One of the difficulties associated with the development of gene therapy is that a therapeutic nucleic acid needs to be delivered to the nucleus inside the cells of a patient. To ameliorate

gene transfer efficacy, one solution is to protect the genetic material into a vehicle that is efficiently captured by the cells, referred to as a gene therapy vector. The target tissue of the treatment determines the type of vector to use for gene transfer. For some applications, for instance expressing a toxic protein in tumor cells, transduction of dividing cells and local transient gene expression is required. For other purposes, such as in IEMs, this is crucial to be able to transduce slowly-dividing cells and obtain a persistent transgene expression. Among other problems that hamper progress of gene therapy, there are the intrinsic toxicity of exogenous proteins, host immune responses directed against the vector products, and efficient delivery to the target site.

Most often used are vectors derived from viruses, that exploit their natural capacity to deliver their genetic cargo into the nucleus. In fact, viral vectors are used in over 75% of gene therapy clinical trials. These vectors carry the genetic material into the nucleus of specific targeted cells. Once there, the therapeutic gene may become part of the host cell's DNA. In this situation, after its integration, the therapeutic gene is transmitted to the daughter cells after cell division. We are then talking about integrative viral vectors. This is the case, for example, of the lentiviral (LV) vectors, which are derived from HIV. The therapeutic gene may stay persist in the host cell, without inserting itself into the host genome. We are then talking about non-integrative viral vector. This is the case, for example, of the vectors derived from adeno-associated virus (AAV). Non-integrative viral vectors are usually considered safer for *in vivo* gene delivery, presenting a low profile of genotoxicity and carcinogenicity. Among various viral-based vector systems, LV and AAV vectors have shown the greatest clinical success for *ex vivo* and *in vivo* gene delivery, respectively (**Figure 1.14**).

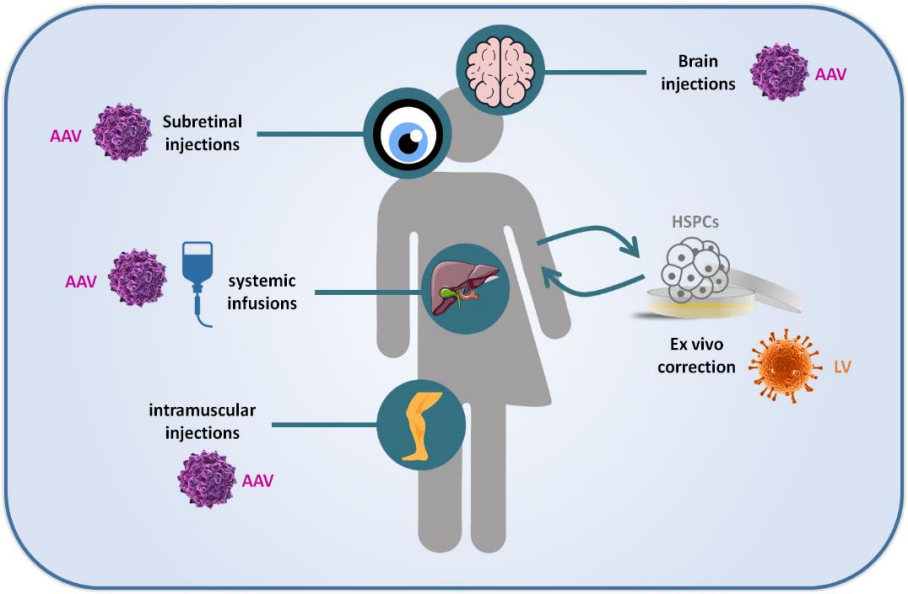


Figure 1.14 In vivo or ex vivo gene therapy approaches.
In vivo approach consists in the injection of vectors delivering the therapeutic gene (or the genome editing tools) into the patient. According to the tissue to be targeted, the injections can be performed by different routes such as systemic intravenous administration, local brain administrations (e.g., intracerebral, intracerebroventricular, and intracisternal injections), retinal (subretinal or intravitreal injections), and muscle (intramuscular) injections. The ex vivo appraoch involves the removal of target cells from the patient and their culture in vitro followed by the correction of the genetic defect by viral vectors encoding the gene of interest (or the genome editing tools) and reinfusion back into the patient. Adapeted from Brunetti et al., 2020[281]

In parallel, non-viral techniques are continually ameliorated to deliver genes and nucleoprotein complexes (mainly for genome editing purpose). These non-viral vectors consist of synthetic or naturally occurring chemical compounds that interact with RNA or DNA. They present many advantages, such as safety and simplicity of use. They allow the transport of relatively large piece of genetic material, their production is inexpensive compared to that of viral vectors, and they are weakly immunogenic. The main non-viral delivery carriers used are cationic lipids – lipoplexes, cationic polymers – polyplexes (such as poly-L-lysine, polyethylenimine, etc.) or cationic proteins, able to create electrostatic interactions with anionic DNA (**Figure 1.15**). The role of these molecules is to protect the genetic material from degradation and to facilitate the delivery into the target cells by endocytosis. Unfortunately, these approaches generally remain less effective than those that use viral vectors, especially in the case of systemic *in vivo* treatment.

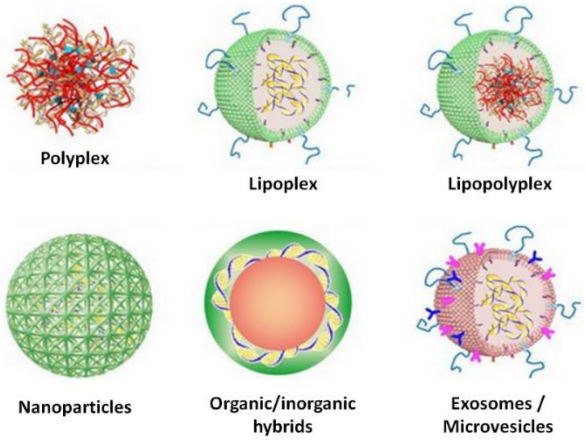


Figure 1.15 Types of non-viral vectors for gene delivery.
From Zhou et al., 2017[284]

In terms of *ex vivo* approaches, electroporation and nucleofection, in which an electrical field is applied, are widely used, particularly for the delivery of genome editing tools (proteins and oligonucleotides). Over the years, a number of methods to deliver non-viral vectors, such as nanoparticles, have been developed and have been the subject of many reviews underscoring that the non-viral vectors strategy continues to progress.[285-288] In particular, promising results were obtained in two mouse models of MMA after administration of LNPs encapsulating therapeutic mRNA (mRNA-3704)[247, 256] and a phase I/II clinical trial is ongoing using this technology. Non-viral vectors will not be more detailed in this manuscript.

The ideal vector for gene therapy in IEMs should be able to contain a large expression cassette with all the elements needed to drive a specific and persistent gene expression to the desired target cell-type. With a low immunogenic profile and a limiting toxicity, it should be safe after administration in patient. The manufacturing process should be easily scalable to facilitate production of large amounts of vectors for reasonable costs and should be in line with the high quality and safety standards required for clinical application. The final product should be contaminants-free and stable enough for long-term storage. Such a perfect vector does not exist and each vector developed so far has its own advantages and drawbacks. These challenges led researchers to develop new strategies and as a result, the past years have witnessed the advent of many innovative approaches.

Gene replacement therapy

Ex vivo approach

Ex vivo approach allows to better control the different steps of the gene transfer, to use less vectors and to avoid dispersion of the treatment in non-target organs. This solution is most often used for the treatment of blood diseases, as it is possible to remove the cells to be corrected by a blood sample. It is based on the autologous transplantation of genetically modified hematopoietic stem/progenitor cells (HSPCs). In contrast to allogenic HSCT requiring a compatible donor, autologous *ex vivo* gene-corrected HSPCs transplantation represents a safer treatment option because it abrogates the risk of major complications such as graft versus host disease and immune rejection. E*x vivo* protocols are usually performed with a pre-transplant conditioning (such as with a busulfan myeloablative regimen) to create a space in the bone marrow niche and facilitate the engraftment of transplanted HSPCs.

LV-mediated gene transfer is nowadays the procedure of choice to deliver multiple copies of a therapeutic gene into human HSPC, as considered by far the safest and most efficient tool. This approach can even now be considered as effective, based on results of clinical gene therapy trials in patients with hemoglobinopathies or immunodeficiencies.[274, 289, 290] Consequently, the first *ex vivo* gene therapy drug (Strimvelis®, GSK), was introduced in 2016 for patients with severe immune deficiency (ADA-SCID).

Exploiting the capacity of the genetically corrected myeloid cells to cross the BBB and differentiate into microglia, *ex vivo* gene therapy has been also successfully applied for the treatment of two IEMs associated with neurological damages, XL-ALD and MLD.[275, 276, 291] Clinical positive results for XL-ALD granted the breakthrough therapy designation from the FDA to the gene therapy product (Lenti-D) in May 2018 and a phase III trial has been recently opened (January 2019, NCT03852498). Overall, these studies demonstrated safety and efficacy and support the application of similar HSPCs-based *ex vivo* gene therapy in other neurodegenerative diseases. Unfortunately, some of these disorders such as MLD, are not yet included in newborn screening programs and in consequence, the affected children would not be diagnosed at the pre-symptomatic stage of the disease, and will not be able to completely benefit this effective therapy. Under pressure from patients associations (particularly from the Aidan Jack Seeger foundation), XL-ALD was added to the newborn screening program RUSP in 2015, allowing identification of the pre-symptomatic at risk patients.

MPSs were also studied as candidate for *ex vivo* gene therapy application through multiple preclinical investigations, based on the transduction of HSPCs with γ-retroviral and LV vectors.[292-297] Notably, transduction of autologous HSPCs with a therapeutic LV resulted in a sustained over-expression of the deficient enzyme, especially in the brain, a normalization of lysosomal storage, and correction of the neurological and systemic manifestations of the disease. This approach was recently translated to the clinic by different investigators in Europe and phase I/II studies for children with MPS I and MPS III are currently ongoing (NCTs 03488394, 04201405). Promising preliminary results concerning the first patients were presented during the last annual meeting of the American Society of Gene and Cell Therapy (ASGCT), in May 2020, showing sustained expression of the deficient enzyme in plasma and leukocytes and correction of the accumulated substrate in the different compartments including CSF.

Other recent encouraging results suggest that Cystinosis could also profit from this therapeutic solution.[298] Indeed, positive trends for some important kidney functions and more than 80% of reduction of accumulated substrate in lysosomes were also reported at the last ASGCT meeting, for a first patient treated by *ex vivo* gene-corrected HSPCs (Avrobio Inc., ongoing phase I/II clinical trial NCT03897361).

In parallel, as an alternative to LTx in IEMs, *ex vivo* gene therapy of autologous hepatocytes was also developed during these last years. This strategy presents the interesting advantage to eliminate the need of life-long immune suppression. The first attempt was performed in 1992 in a patient with FH. While it was a success in term of feasibility, the therapeutic efficacy was disappointing. The trial resulted in transient and partial correction of the disease. Several hurdles were pointed as the need of invasive procedures to obtain hepatocytes, the difficulty to maintain them in culture, their limited engraftment and their reinfusion in the portal vein following *ex vivo* gene replacement.[299] In fact, the only pathologies that can be candidates to liver-directed *ex vivo* gene therapy are those which do not need high levels of transgene expression and with limited liver damages. Hence, this approach was explored in metabolic pathologies such as CN syndrome, with mitigated results.[300-302]

The safety in the use of integrative vectors is a major concern for the *ex vivo* approach, and the risks of genotoxicity and carcinogenicity have to be carefully evaluated. To limit these risks, a specific vector design is needed.[303] For example, the usage of promoters of human origin, characterized by a short-range activity, and driving physiological transcriptional levels are strongly recommended for gene therapy applications.

In vivo approach

Given the limitations of *ex vivo* gene transfer, *in vivo* gene therapy represents a valuable alternative for different IEMs such as disorders affecting muscle, CNS, respiratory functions, eye, or heart. As previously mentioned, it is defined as the direct delivery of a therapeutic gene into the body, in order to achieve a long-term therapeutic benefit. According to the tissue to be targeted, the vector can be delivered by different routes such as systemic intravenous administration, local brain administrations (e.g. intracerebral, intracerebroventricular, and intracisternal injections), retinal (subretinal or intravitreal injections), and intramuscular injections (**Figure 1.14**).

A large number of vectors have been developed and investigated, but so far the AAV vectors or recombinant AAVs (rAAVs), have emerged as the most promising tools for *in vivo* gene transfer, based on their remarkable transduction efficacy and safety profile, that have been confirmed also in human trials. rAAVs are derived from small, non-enveloped, and non-integrating AAVs. Their biodistribution and the consequent specificity of transgene expression depends on the viral capsid serotype, route of administration, and vector design. Considering the natural tropism of AAVs and the unmet medical needs, most rAAV gene therapy programs have been focused on the liver, skeletal muscles, eye and the CNS. rAAVs have the capability to transduce non-dividing cells and achieve long-term expression of the transgene with low toxicity but are limited by a small cloning capacity, the encapsidated genome size being only near 4.7kb. Their development has strongly increased in the last decades and since the first clinical trials in the 1990s, there have been over 180 gene therapy trials with AAV vectors (Search of: AAV | Interventional Studies – ClinicalTrials.gov). Currently, there are 116 clinical trials ongoing, involving 81 products for the treatment of 40 diseases (**Table 1-2**). Finally, several rAAV-based products have recently reached the

market (Glybera® from 2012 to 2017, Luxturna® in 2017 and Zolgensma® in 2019, **Figure 1.12).**

Unfortunately, the clinical use of rAAVs is limited, notably due to natural exposure to wild-type AAV, with a large number of individuals that possess antibodies against the capsid proteins.[304-311] In fact, the viral capsid of rAAV is identical or nearly identical to the capsid of the wild-type virus and the presence of anti-AAV neutralizing antibodies (NAbs) prevents gene transfer.[270, 312-315] Accordingly, the presence of these specific antibodies is now a common exclusion criterion for enrollment into clinical trials. Moreover, the vector triggers immune reaction, leading to high titer of NAbs thus restricting its use to a single administration.[316]

Table 1-2 Diseases for which clinical trials using AAV-Based gene therapy products are ongoing

Diseases
Cardiovascular Diseases
Critical Limb Ischemia (CLI)
Refractory Angina
Hematological Diseases
Hemophilia A
Hemophilia B
Infectious Diseases
HIV-1 Infected Adults with Controlled Viremia
Inherited Metabolic Diseases (excluding Lysosomal Diseases)
Crigler Najjar Syndrome
Glycogen Storage Disease IA
Homozygous Familial Hypercholesterolemia
Ornithine transcarbamylase deficiency
Phenylketonuria
Lysosomal Diseases
Batten Disease
Danon Disease
Fabry Disease
GM1 Gangliosidosis
Mucopolysaccharidosis I
Mucopolysaccharidosis II
Mucopolysaccharidosis III
Mucopolysaccharidosis VI
Pompe Disease
Neurological/Neurodegenerative Diseases
Alzheimer's Disease
Aromatic L-amino Acid Decarboxylase Deficiency
Charcot-Marie-Tooth Neuropathy 1A
Giant axonal neuropathy
Huntington's disease
Parkinson's disease
Neuromuscular Diseases
Duchenne Muscular Dystrophy
Limb Girdle Muscular Dystrophy E
Spinal muscular atrophy
X-linked myotubular myopathy
Ophthalmological Diseases
Achromatopsia
Age-related Macular Degeneration
Choroideremia
Leber Congenital Amaurosis
Leber Hereditary Optic Neuropathy
Retinitis pigmentosa
X-linked retinitis pigmentosa
X-linked juvenile retinoschisis
Miscellaneous
Ageing
Irradiation-Induced Parotid Salivary Hypofunction/Radiation-induced Xerostomia
Osteoarthritis of the Knee

To get around this problem and overcome this humoral immune response, researchers diversified their capsid to increase the variety of serotypes and combine immunomodulation with gene therapy (e.g. plasmapheresis, pharmacological modulation of the B cell, and/or T-cell activation, see more details in the further section **New development to unlock the potential of AAV-based LGT**).[317-319] In addition, a cytotoxic T lymphocyte (CTL)-

mediated cellular immunity directed against the AAV capsid was highlighted during the first clinical trials of AAV-based gene therapy[269, 270, 320]. Since, the scientific community has focused on its potential impact on the gene transfer efficacy.[310, 311, 321, 322] The clinical use of glucocorticoids in patients appeared efficient to stop this (CTL)-mediated immune response.[271, 272]

Being a natural target tissue of AAVs, the muscle has been the fisrt for *in vivo* gene therapy because of its simple access through intramuscular injections. After encouraging results obtained in Hemophilia B animal models (ref mice/dogs), this approach was translated into human, showing evidences of safety and efficacy.[314, 323, 324]

In addition to correcting muscle tissues, the muscle is also an attractive target for delivery of secreted proteins into the circulation after gene therapy, given its secretory capacity.[325, 326] For example, in patients with lipoprotein lipase (LPL) deficiency, while intramuscular administration of AAV1 vectors was associated with a transient CTL immune response against AAV capsid proteins, gene transfer was well tolerated and led to a sustained disease correction when associated with immunosuppressive treatments.[327-329] The results of this trial led to the official approval by the European Medicine Agency (EMA) of Glybera® (also named alipogene tiparvovec) as the first gene-therapy medicine in the European Union.[330]

Up to now, several gene therapy approaches were developed for a variety of myopathies, especially those affecting the entire body (e.g. DMD, X-linked myotubular myopathy (XLMTM), limb girdle muscular dystrophies (LGMDs), etc.), but also for IEMs such as GSDs or AATD. In particular, the different approaches attempted for PD (GSD II) will be detailed in a next chapter. Despite all these interesting different approaches, it has been particularly challenging to achieve robust and widespread expression of a given therapeutic gene in the skeletal muscle.[325, 331-333]

For instance, clinical trials for DMD have established proofs that targeting the muscle by gene therapy is feasible, though the overall therapeutic benefits were limited.[331, 334] Though increasing the vector doses may result in more efficient gene transfer, this concomitantly increases the risk of triggering inadvertent immune reactions.[335, 336] Indeed, even if AAV vectors elicit only a weak innate immune response, recent studies suggested that this response may occur with high vector doses, affecting the safety of this approach. In fact, several adverse events have occurred in patients receiving high dose systemic AAV and have resulted from innate immune responses (e.g. inflammation and complement activation) and cellular immune responses to vector few days/weeks following intravenous administration. First alerts were given by the detection of severe toxicities in piglets and juvenile non-human primates after administration of high doses of an AAV9.[337] Then, a clinical trial for DMD was placed on hold by the FDA after the report of a patient presenting strong reduction of platelets and red blood cells, transient renal impairment, and complement activation, several days after administration of high dose of an AAV9.[338] Nevertheless, this patient did not show signs of bleeding, clotting abnormalities, or liver dysfunction, recovered from the event, and the trial was resumed. But more recently, in a clinical trial for XLMTM (ASPIRO, NCT03199469), three young children tragically died from liver failure associated with internal bleeding and sepsis, few days following a high dose of AAV8 injection.[339] Investigations to determine whether this was related to treatment with a high-dose AAV therapy are still ongoing, but such large viral doses could certainly cause toxicity, especially in a damaged liver, as it seems to be the case in these two young XLMTM boys. To note, a similar dose of AAV9 has been administered in pediatric patients in a clinical trial for SMA (NCT03461289, see details below) without reported serious treatment-related adverse events. Thus, a threshold of toxicity for systemic AAV probably exists and it will be safer

for future clinic trials to precisely determine this threshold. Hence, this drama requires developing more potent gene therapy vectors so we don't have to use such high doses, focusing efforts on notably i) novel promoters and regulatory elements that outperform conventional ones and that consequently allow for high and widespread muscle-specific expression at lower and safer vector doses; ii) engineered capsids that better home to muscle; iii) safer manufacturing methods, reducing impurities (host proteins and undesired nucleic acids); iv) innovative strategies to avoid immune responses.[340] These events clearly indicate that careful oversight and management of immune responses (adaptive and innate) and liver monitoring should be included in all clinical protocols.

Despite this tragedy, considerations on the clinical/benefit ratio support the fact that systemic administration of high-dose of AAVs was a safe and efficient method of gene therapy particularly in life-threatening diseases such as spinal muscular atrophy (SMA). This disease represents a good example of what gene therapy can currently achieve in situations of major therapeutic deficiency. Systemic administration of high AAV9 vector doses, the treatment has improved motor function in 15 children (NCT02122952).[341] By 24 months following infusion, none of the 12 patients of the high-dose cohort required permanent ventilation and nine of them reached the ability to sit without support for more than 30 seconds. More impressively, two patients reached the ability to stand and walk independently. These results showed a dose-response relationship. The results of this trial led to the official approval by the FDA of Zolgensma® in May 2019.

A large part of rAAV gene therapy under clinical development is also focused on ocular diseases, such as Leber's amaurosis, Leber's optic neuropathy or Retinitis pigmentosa. These works were reviewed very well these last years, providing details of experimental challenges and critical benchmarks.[342-344] The eye, easily accessible, is a compartmentalized organ that is amenable to direct ocular delivery. One treatment (Luxturna®, voretigene neparvovec), that was the first rAAV gene therapy drug approved by the FDA, is already available for Leber's amaurosis, consisting of an AAV2 injection directly into the retina of affected patients, and resulting in an improvement of the visual capacity.[345]

CNS, and especially the brain, is much more complex to target, but several strategies were proposed to offer efficient gene therapy solutions for neurological disorders. For application in IEMs, particularly in LSDs, rAAV are commonly injected directly into the brain by intraparenchymal injections, or into the CSF by intracerebroventricular (ICV), cisternal or lumbar-intrathecal administrations. The first route is particularly suited for diseases that afflict a defined region of the brain (such as the putamen in Parkinson disease for example), and require a localized distribution of rAAV. By targeting the CSF, a broader distribution can be achieved. Administered through these routes, rAAVs can efficiently target neurons, astrocytes, oligodendrocytes and microglia.[346] Presenting the advantage to bypass the BBB, these routes of administration remain very invasive and are not without risks for the patients. As an alternative, systemic delivery of certain serotypes, such as AAV9 and AAVrh.10, was demonstrated as efficient to transduce neurons and glia, crossing with success the BBB.[347-350] Several studies were performed with success for diseases that afflict widespread regions of the CNS, (e.g. SMA, amyotrophic lateral sclerosis (ALS), late infantile CLN (LICLN), Canavan disease, GM-1, MPS III, spinal cord axonopathies, adrenomyeloneuropathy (AMN) etc.)[351-361] and several clinical trials are currently underway (NCTs 01161576 and 01414985 for LINCL, 01801709 for MLD, 01474343 for MPS III or also 02362438 for Giant axonal neuropathy (GAN)).

However, because of leakage of the system, local rAAV administration into the CNS results in transduction of non-target tissues and peripheral transgene expression, in particular in

the liver.[282, 346] This may lead to the development of an immune response, limiting the benefit of the *in vivo* gene transfer strategy for these applications. To overcome this limitation, different strategies were developed such as the use of transient immunosuppression and tolerance induction.[305, 311, 362] In particular, strategies inducing liver-mediated tolerance or neonatal AAV-mediated systemic transgene expression before CNS-directed gene transfer are encouraging.[363-365]

To finish, this rapid overview of existing *in vivo* gene replacement strategies could not be exhaustive, without mentioning the remarkable results obtained with this approach in the field of hematological diseases. Indeed, the results of two trials for Hemophilia A and B recently published at the end of 2017 are extremely conclusive: they appear to have cured patients.[272, 366] To date, follow-up of these studies confirm the persistence of the therapeutic effect over time and the safety of the rAAV treatment. Here, liver was the target tissue of the gene transfer. The current success of these trials has sparked a great enthusiasm for future developments of liver-directed gene therapy for IEMs. A next section will be dedicated to this particular liver gene therapy platform.

Before turning to the other gene therapy strategies, it seems essential to evoke in this section the potential risk of genotoxicity of AAV. The careful assessment of this crucial parameter is becoming a new safety requirement of competent regulatory authorities. It is described that AAV vector genomes have a low frequency of integration into the host genome and only a minor proportion may be randomly inserted.[367, 368] Consequently AAV vectors are associated with a low risk of genotoxicity.[369-371] The potential AAV vectors link with tumorigenesis is controversial and still debated today. Evidence that AAV genome integrations could lead to the formation of hepatocellular carcinoma (HCC) was initially provided in experiments performed in mice (MPSs, MMA mouse models).[372-374] In this studies, a higher incidence of HCC was observed at higher vector doses, with constitutive promoters or promoters with high transactivating activity, and in neonate mice. The mechanism postulated for the induction of HCC in mice via AAV vector integration was the dysregulation of microRNA-341 (Mir341) proximal to the Rian locus. Indeed, the Mir341 resulted to be located in the mouse genome in a hotspot for AAV genome integration. However, this locus has no orthologues in humans.[374] In another study performed in adult mice, no evidence of insertional mutagenesis and cancer was documented in a follow up study of 18 months.[370] Similarly, several long-term studies with large animal models such as dogs and non-human primates indicated a relative safe profile of AAV with low integration.[269, 375] Moreover, no instance of HCC development has been documented in patients to date.[271] More recently, clonal insertion of AAV genome fragments was reported in Hemophilia A dogs injected with AAV vectors expressing clotting factor VIII under the liver-specific TBG promoter.[376] The insertion occurred in genes previously associated with growth control and transformation in humans, but histological analysis did not showed any tumor foci. In human liver, wild-type AAV fragments were found integrated in proximity of known cancer-related genes and a potential involvement in the pathogenesis of HCC was reported.[377, 378] These integrated fragments corresponded to transcription factor-binding sites that could play the role of enhancer-promoter elements, thus presenting a risk of transactivating activity.[379] Of note, this sequence is present in the first generation of transgene expression cassettes used in AAV gene therapy. No information concerning rAAV has been documented to date and given that the number of AAV-treated subjects remains small, further studies are necessary to clarify the impact of AAV gene therapy on HCC development together with a careful follow-up in injected subjects.

Gene addition

By gene addition, rAAV-mediated gene therapy has the potential to address complex diseases that are unmet medical needs, such as heart failure and infectious diseases. It can act by supplying neurotrophic factors for neurological diseases[346] or interfere in different signaling pathways for heart failure and cancer.[380, 381] This platform is also being tested to deliver genes encoding antibodies that can neutralize severe viral infections (such as Influenza, HIV or Corona viruses)[382-388] or nanobodies that can rebalance homeostasis.[389] Following these approaches, muscles or liver are used as bio-factory to produce therapeutic antibodies that are secreted into the bloodstream. This strategy was tested clinically for HIV infection (NCT01937455) demonstrating that neutralizing responses to HIV can be induced using vectored immuno-prophylaxis with AAV, and this approach might be feasible for infectious disease prevention.[390] Here, the challenge of future studies will be to overcome the development of anti-drug antibody (ADA) response that have diminished HIV antibody expression, neutralizing the circulating therapeutic antibody.[391] Different HIV antibodies and different AAV serotypes might improve transduction and expression after intramuscular administration.

Gene silencing

Gene silencing is intended in particular for diseases caused by gain of toxic function mutations, such as Huntington disease. Given their potency for inhibiting gene expression, RNAi strategies (see **RNA-targeted therapeutics** section) largely dominate rAAV-based approaches that are still under preclinical development.[392] Main difficulty for AAV-based gene silencing lies in the level of transduction to achieve enough therapeutic efficacy without leading to toxicity, which is still a challenge for AAV vectors with certain target organs.[393] Nevertheless, this approach may be of interest for IEMs that can be targeted by SRT. Indeed, by limiting the deleterious accumulation of a toxic metabolite, AAV-based gene silencing could bring a novel option for disorders that do not benefit yet from available SRT drugs.

Close to the gene editing strategies, a recent approach describes the use of Cas13 family proteins to silence gene expression at the mRNA level.[394, 395] These proteins, by the help of guide RNA (gRNA), have an efficient ribonuclease activity and seem to be more specific than RNAi. However, their translational use still faces several obstacles including rAAV packaging size limitations and immune responses against the bacteria-derived proteins.[396, 397]

Gene editing

As indicated by its name, this technique allows DNA correction. In other words, this strategy inserts, removes, changes, or replaces specific pieces of DNA, with the goal to change the existing gene and correct mutations where they occur. Gene editing can be performed both *ex vivo* and *in vivo* and presents advantages that gene replacement therapy has not, such as i) a physiologically regulation of the corrected gene expression, ii) the possibility to target autosomal dominant diseases due to gain-of-function mutations, or iii) the persistence of the DNA modification in proliferating cells, overcoming a dilution effect.

This strategy has been studied since the 1980s and several types of editing tools have been developed so far. It typically occurs in two steps: generation of targeted DNA breaks into

the genome and DNA repair that leads to a desired DNA alteration. To generate DNA breaks, multiple nucleases can be used, such as engineered mega-nucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the most recently discovered clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) proteins. Each system possesses pro and cons. For example, ZFNs are tolerable because they are derived from human proteins, and their small transgene sizes are amenable to rAAV packaging. In contrast, the Cas proteins are derived from bacteria, resulting in potential immunogenicity in particular after *in vivo* use and their large transgene sizes make rAAV delivery more difficult. However, Cas proteins are a robust system, and their large flexibility and strong efficiency are a major advantage over other nucleases. For this reason, Cas proteins are, nowadays, the most extensively studied programmable nucleases for both research and therapeutic applications. The main cellular DNA repair pathways are the non-homologous end joining (NHEJ) and homology-directed repair (HDR) that are exploited to introduce gene disruptions and precise corrections, respectively. For repair by HDR, a template of the corrected sequence has to be used as a substrate and is in consequence co-delivered with the nuclease transgene (**Figure 1.16**).

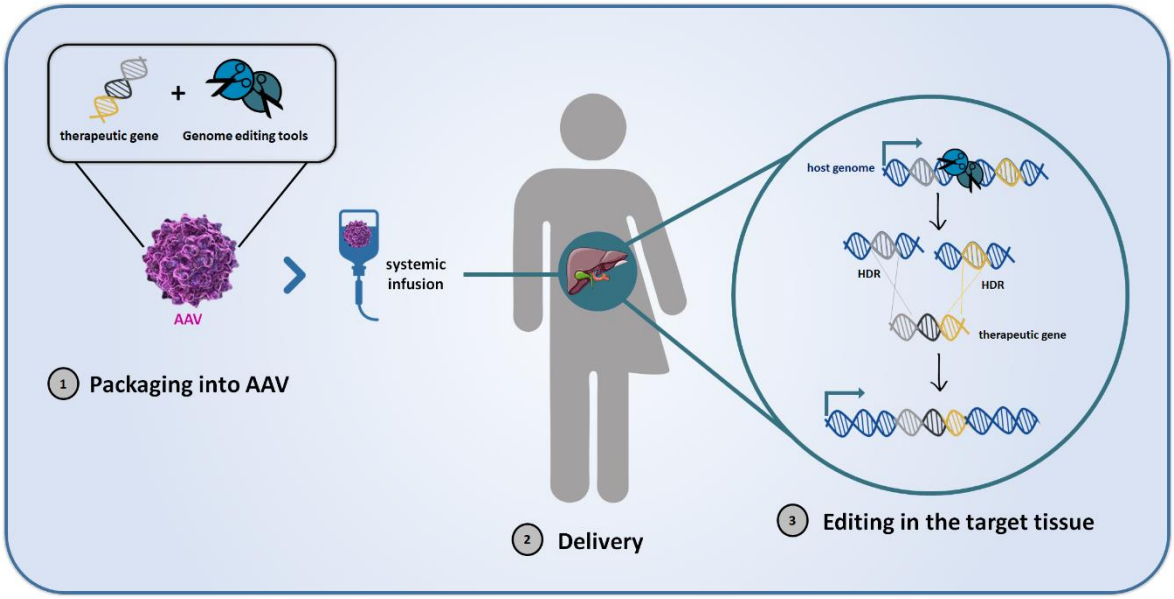


Figure 1.16 In vivo genome editing
(1) Donor DNA sequence and the genome editing tools (ZFNs, TALENs, or CRISPR/Cas9) are packaged into AAV vectors. (2) one time systemic infusion, the AAV vectors achieve their target tissue. The liver was used here as example.(3) Editing tools bind specifically to the host genome, induce double-strand break that stimulates homology-directed repair (HDR) for replacement of a mutated gene or targeting gene insertion at a specified locus (e.g. albumin locus). Adapeted from Sangamo Therapeutics Inc.©

This powerful strategy has the potential to generate a therapeutic approach for a large number of diseases.[398-406] As illustration, one can mention the first three *in vivo* rAAV gene-editing clinical trials that are ongoing for Hemophilia B (NCT02695160), MPS I (NCT02702115) and MPS II (NCT03041324). In these specific examples, the ZFN platform and HDR pathway are used to insert a therapeutic gene into the albumin locus to hijack the strong albumin promoter in order to drive the transgene expression in hepatocytes.[407-409] Among other examples of application including exon deletion, splicing modulation and sequences exchange,[410-413] one can highlight the first study demonstrating the feasibility of gene editing to restore dystrophin expression in a canine DMD model.[414]

If genome editing tools have been largely delivered by AAV vectors in preclinical studies,[415] interesting alternatives are LNPs, which can also deliver drugs or nucleic acids to various tissues, including liver.[403] Indeed, one limitation of the AAV-based delivery system is the long-lasting expression of the editing machinery that can lead to DNA damages, improper gene editing, deleterious cellular effects that can degenerate into cancer development. Although not observed in preclinical animal models, these consequences are of course serious concerns.[416, 417] Another major limitation of genome editing is the specificity of action and the possibility of off-target cleavage that might have serious effects on clinical safety.[418]

Despite these concerns, genome editing has an enormous potential and is rapidly advancing into the clinic. Several approaches are under investigation for many different diseases such as HIV infection resistance,[419] β-thalassemia (NCT03432364),[420] leukemia and solid tumors.[421] Although there are no approved treatments available at this time, the ongoing clinical trials will allow to further investigate the safety and the efficacy of this approach.

Another attractive application perfectly illustrates the great potential of this platform. It has been reported a proof-of-concept study for the silencing of an extra chromosome in pluripotent stem cells from a patient with Down syndrome.[422] Of course to date, applying this strategy to the whole organism is a major but exciting challenge.

This field of genome editing is booming and the new generations of tools are progressing very fast, improving greatly the editing efficiency, accuracy and flexibility,[416, 417, 423] opening very interesting therapeutic perspectives. In fact, this approach have the potential for becoming the next generation of gene therapy for IEMs when technological limitations, such as off-target effects, immune reactions and ethical issues will be addressed.[424]

CAR-T cell therapy

CAR T-cell therapy is a particular strategy that combines cell and gene therapies. It involves changing immune cells to recognize and fight other specific cells inside the body. This approach has the potential to treat many different conditions such as cancer, autoimmune diseases, and neurologic disorders. It may also be used to rebuild damaged cartilage in joints, repair spinal cord injuries, and strengthen a weakened immune system. Basically, some T cells from affected patients are modified by *ex vivo* gene replacement therapy in order to confer them a particular receptor, called a chimeric antigen receptor (CAR), capable to bind specifically to the target cell. After binding, the CAR activates an intracellular pathway that lead to immune cell activation and elimination of the target cell (**Figure 1.17**).

Designed for the treatment of malignant blood diseases, two first drugs developed with these modified T-lymphocytes were approved by the FDA in 2017: Kymriah® and Yescarta®.[425-428] These successes make it possible to consider the emergence of new CAR T-cell therapies for other applications and for example directed against solid tumors. However, potentially fatal toxicity including cytokine release syndrome and neurotoxicity indicate that the technology needs further developments.[429] The next generation of CAR T-cells will aim at a better control of their proliferation on improved functions through the implementation of inducible inactivation mechanisms. Affinity fine-tuning, multi-CAR T-cells, and combination with immune checkpoint inhibitors open new opportunities. Finally, synthetic biology approaches leading to personalized cell therapy marks the beginning of a new area for immunotherapy.[430]

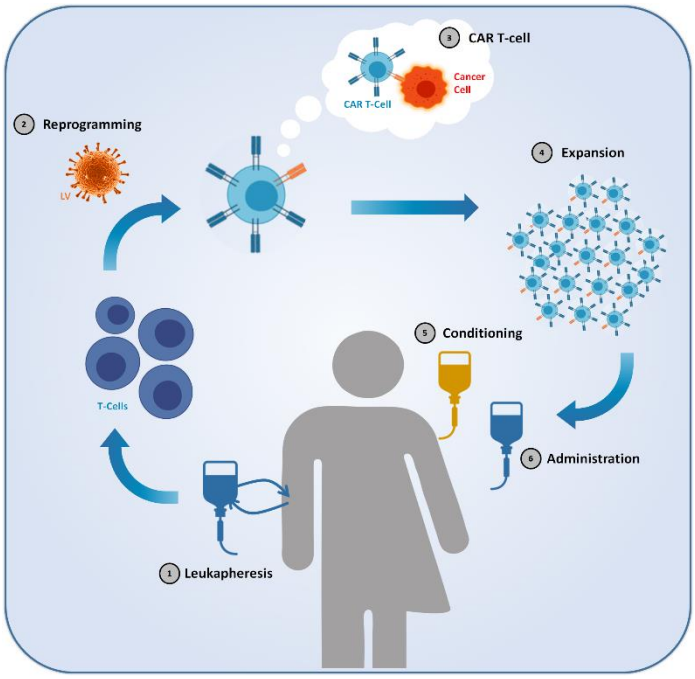


Figure 1.17 CAR T-cell therapy
(1) Through a process called leukapheresis, blood of a patient is collected and T-cells are separated out. The rest of the blood is returned to the body. (2) The T-cells are reprogrammed by lentivirus (LV)-mediated gene therapy so the T-cells produce a specific surface chimeric antigen receptor (CAR). (3) The CAR T-cells can now recognize and attach to specific marker proteins of the cancer cell surface, signaling its destruction. (4) The CAR T-cells are then expanded in vitro to create millions of clones. (5) Before the CAR-T cells administration, the patient undergoes conditioning chemotherapy. (6) Finally, CAR-T cells are infused back into the patient. Adapted from Malaghan Institute of Medical Research ©.

2.2 AAV-based liver gene transfer

The liver is a particularly attractive organ for the development of gene therapy. One of the reasons is the natural capacity of this organ to produce and secrete molecules in the bloodstream, and it has the role of bio-factory that can be exploited by the gene therapy approach. The liver is very well vascularized, allowing an easy access for the therapeutic vector after systemic injection. Furthermore, different studies in small- and large-animal models and in humans have demonstrated that it is possible to efficiently transduce hepatocytes and achieve long-term stability of the gene transfer.[269, 271, 272, 366, 375, 431-433] In addition, the liver presents an important advantage from an immunological perspective. The expression of a transgene in hepatocytes induces antigen-specific tolerance mediated by regulatory T cells and other mechanisms (**Figure 1.18**) thus limiting the development of a detrimental immune response against the therapeutic transgene. [318, 434-436] Liver-directed gene transfer (LGT) is an attractive strategy for the treatment of a variety of IEMs.

As for LTx, one important parameter to take into account is the presence or the absence of liver parenchymal damage underlying the disease and the its progression. Indeed, when liver structure and hepatocyte functions are normal, the replacement of the defective enzyme or circulating protein may be more easily performed and can successfully treat or prevent the systemic manifestations. This appears quite more challenging in IEMs characterized by liver damages and hepatic injury, where the impaired liver architecture could limit the gene therapy efficacy and increase the risk of toxicity.

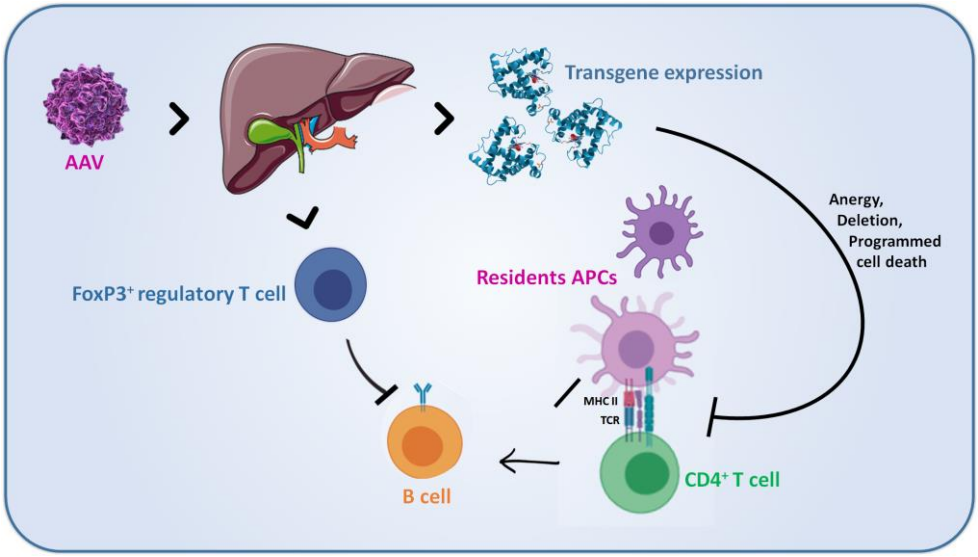


Figure 1.18 Induction of hepatic tolerance with AAV vectors
Liver-directed gene transfer with AAV vectors induces immunological tolerance via multiple mechanisms. These include the deletion of reactive CD4+ T helper cells (e.g., by programmed cell death). Induction of FoxP3+ regulatory T cells plays a central role in the induction of hepatic tolerance. Similarly, antigen presentation by resident APCs in the liver draining portal and celiac lymph nodes is key to the induction of hepatic tolerance. MHC II, major histocompatibility complex class II; TCR, T cell receptor; BCR, B cell receptor; AAV, adeno-associated virus vectors; APC, antigen presenting cell. Adapted from Ronzitti et al., 2019[437]

An appropriate timing for LGT is also crucial. A classical example is the OTC deficiency. This disease manifests by metabolic crises at early age (soon after birth), during childhood, or in adulthood. During these crises, the liver suffers from an acute toxic injury that leads to hepatocytes necrosis and regeneration, conditions that are known to reduce the efficacy of gene transfer with non-integrative vectors as AAV. As OTC deficiency, several IEMs have an acute onset in the newborn period and successful treatment for these severe neonatal disorders is considerably difficult. Another relevant aspect of LGT with vectors that do not integrate in the host genome, is linked to the fact that the liver grows progressively during childhood and possesses a capacity of regeneration after a damage. For this reason, an early LGT realized soon after birth (often the ideal timing to correct a severe IEM) ends up with loss of efficacy caused by the loss of episomal vector DNA in the transduced cells in a rapidly growing liver mass.[438-441] That's why with LGT strategy, participants recruited to clinical trials have been largely limited to adults with fully developed liver. An alternative could be the possibility to re-administer the AAV vector, and therefore, the development of strategies that prevent the development of a humoral immune response is urgently required (see more details in the next section **New development to unlock the potential of AAV-based LGT**) .[305]

Despite these limitations, evidence of gene transfer efficacy were provided with AAV-based LGT in animal models and in humans of various IEMs (e.g. Hemophilia, FH, AATD, HT-1, UCDs, GSD Ia, WD, OAs, PKU, FAODs, CN syndrome, AIP, PFIC, PH1, etc.).[440-484] They demonstrated that almost several AAV capsids can transduce liver efficiently following systemic administration and they showed long-term correction. Based on their safety and efficacy also clinically proved, rAAVs have emerged as robust and excellent tools for LGT.[280, 281, 283, 442, 485, 486] Nowadays, while important differences in transduction efficacy exist between animal models and human livers, intense research to improve LGT and identify more potent and efficient AAV vectors is ongoing.[487, 488]

Clinical studies in IEMs

After years of efforts, AAV-based LGT reached the clinic and the results support safety and efficacy of the approach.[270-272, 366, 432, 433, 489] The first trial, based on AAV2 vector administration in severe Hemophilia B patients, was particularly important for the field of *in vivo* gene transfer, as it demonstrated for the first time that it was possible to transduce the human liver with AAV vectors, leading to therapeutic levels of transgene expression.[270] However, what was not predicted in animal models, was the fact that liver expression in humans was not sustained, due to the development of a CTL immune response, that cleared of the transduced hepatocytes.[490] Another important lesson learned from this pioneer trial was that pre-existing humoral immunity to AAV in humans completely prevented liver transduction.

As earlier mentioned, the other major milestone in this field was the successful second clinical trial for Hemophilia B. Several years after intravenous AAV8 administration, a stable expression of the clotting factor IX was observed in seronegative treated patients, resulting in the reduction of bleeding episodes and need for prophylaxis in the group of patients receiving the higher vector dose.[271] In this study, a mild increase of transaminases was reported, possibly due to a CTL immune response that occurred in the weeks following vector administration. As in the first trial, without any medical intervention, this immune response led to a transgene expression loss. However, the use of tapering glucocorticosteroids treatment resulted in the rapid disappearance of the capsid-reactive T cells from the circulation and long-term expression of the therapeutic gene. Similar results were obtained in other trials in both Hemophilia A and B patients, with AAV5 or bio-engineered AAV vectors.[272, 366, 433] These initial studies represent the basis of the LGT clinical success, bringing knowledge on the safety and efficacy of this approach, and the main goal for the field is now to ensure consistency of these results across large patient populations and for many other applications. Besides Hemophilia, there is an increasing number of AAV-based LGT clinical trials under investigation for different IEMs. The most representative ones will be mentioned in this section.

A first interesting example to evoke is the trial using an AAV5 vector in severe AIP patients (NCT02082860). In a surprising way, this trial showed safety but no signs of gene transfer efficacy. In fact, despite the use of similar AAV doses than in previous trials, the liver transduction was not sufficient and the enrolled subjects did not show significant evidence of changes in any monitored biochemical parameters.[489] However, clinical improvements were observed in these patients and two out of eight have stopped standard of care treatment for more than 3 years now.[491]

In the UCDs field, research has focused on OTC deficiency, the most frequent disorder of this family.[475, 481-483] A phase I/II trial is currently ongoing in late-onset patients (adults) with an AAV8 vector (NCT02991144). No safety issues were reported so far. Mild increase on transaminases was observed, but rapidly solved with glucocorticosteroids. Encouragingly, efficacy evidences were showed in two patients, allowing them to discontinue ammonia scavenger medications and to reduce dietary protein restrictions.[492] Nevertheless, this trial was attempted in adult patients and one can underline that the onset of UCDs, such as OTC deficiency, is frequently in the neonatal period, timing that is not today appropriate for a LGT approach. As previously mentioned, the challenges to address here are to achieve therapeutic expression levels with sufficient rapidity and consistency to limit the progressive loss of transgene expression due to the liver growth.[438, 478]

Other similar clinical trials with AAV8 are ongoing for FH (NCT02651675), CN syndrome (NCTs 03223194 and 03466463, that will be discussed in more detail in a future chapter) and GSD Ia (NCT03517085). In this last trial, no serious adverse events have been reported so far and all patients demonstrated a positive biologic response, reflected by a limited hypoglycemia during a controlled fasting challenge. In addition, all patients (n=9) were able to decrease their daily cornstarch use.[493] Long-term data and start of the Phase 3 study are expected for 2021. In all these trials, the goal is to correct the liver metabolic defect by delivering the therapeutic gene to hepatocytes.

Another LGT strategy relies on the use of the liver as a bio-factory in order to produce and secrete in the bloodstream the therapeutic product (enzyme), which can achieve and correct other tissues, such as muscles and CNS. This specific approach has the potential both to provide a source for sustained corrected enzyme production and to induce immune tolerance due to liver-specific expression of the transgene. This particular LGT currently concerns two LSDs: MPS VI and GSD II, for which ERT is already clinically available but presents important limitations. Preclinical data obtained in animal models have provided encouraging results, demonstrating for both diseases, long-term normalization of different tissues and improvement of motor functions after AAV administration.[494-501] Based on these encouraging results, clinical trials have already started (NCTs 03173521, 03533673). The GSD II trial will be discussed with more details in the next chapter dedicated to PD.

Liver genome editing

Some limitations of LGT approaches based on gene replacement strategy, such as the loss of genome copies (due to episomal nature of the AAV DNA and its dilution/loss during hepatocytes proliferation[439, 441, 502]), can be overcome by genome editing. This specific and permanent modification of the genome can indeed represents a viable alternative for different IEMs, specifically those that need early intervention.

Due to its characterictics described above, the liver is an ideal organ to test gene editing-based therapies in animal model of disease. However, early proof-of-concept studies were only partially successful, showing a gene correction frequency in murine hepatocytes far from a therapeutic application.[503-505] The recent discovery and optimization of different powerful gene editing tools, such ZFN and CRISPR/Cas9, had a real impact on the liver gene editing development. After a few years of effort, a ZFN-based approach has recently reached clinical stage and is currently under investigation in Hemophilia B (NCT02695160), MPS I and MPS II subjects (NCTs 02702115 and 03041324).

To cure IEMs, different strategies of liver gene editing have been attempted so far in animal models.[283] Among them, one can cite i) reprogramming metabolic pathways by gene inactivation; ii) editing of genes that confer growth advantage; iii) correction of hepatocytes that do not present growth advantage; iv) correction of the genetic defect by targeting a “safe-harbor locus”.

A first example to illustrate AAV-based liver gene editing approach as a potential treatment for IEMs, is in the FH application. The different gene inactivation strategies employed for this disease have proven to be efficient in different animal models, including non human primates (NHP).[399, 400, 415] For IEMs presenting accumulation of toxic metabolites, knock-out (KO) strategies have also been employed as SRT, such as in HT-1 and PH1.[406, 506] Interestingly with this approach in HT-1, it was demonstrated a selective advantage to corrected hepatocytes that can proliferate and repopulate the liver. It was confirmed in other

studies, using different efficient therapeutic strategies involving HDR and gene correction mechanisms.[402, 460, 506, 507] Similarly, in AATD, the correction of defective gene involved in the disease, PiZ, results in the selective expansion of edited hepatocytes, consequent to their survival advantage, with the improvement of the liver phenotype.[404, 405, 508] Nevertheless, it is important to note that in absence of a selective advantage, the low efficiency of genome editing results in inefficient gene correction and thus fail to reduce disease symptoms. Because severe liver damages, like fibrosis, cirrhosis and HCC development, are specific risks in patients suffering from diseases such as HT-1 and AATD, further improvements are still required to reduce the number of non-corrected hepatocytes and limit off-target nuclease activities.

This is particularly true for other diseases that do not present selective advantage of corrected cells. Among these diseases, promising results were recently obtained for OTC, using a dual AAV system delivering the CRISPR/Cas9 compounds necessary to correct the mutation. With this strategy, when treated as neonates, mice presented high percentage of corrected hepatocytes, resulting in a therapeutic effect.[398] Importantly, in those animals the levels of Cas9 decreased over time, consequent to liver growth and viral DNA dilution. This study was one of the firsts to provide evidence of the efficacy of an *in vivo* gene editing therapeutic approach in a neonate mouse model of disease, in which the corrected hepatocytes do not have any selective advantage. However, in the same study, when OTC-deficient mice were treated as adults, the efficiency of gene correction was lower and, unexpectedly, resulted in a worsening phenotype.[398] This phenomenon was explained by the limited efficacy of the homologous recombination in non-replicating cells and continuous cutting and repairs occurring at the Otc site, leading to inactivation of the gene. These results confirm that homologous recombination in adults is a major challenge in the field and requires further improvements to achieve therapeutic levels.

As early mentioned in this manuscript, important limitations of AAV-based gene editing strategy are the long-lasting expression of the editing machinery and the genome editing off-target cleavage that can lead both to genotoxic consequences.[418] In addition, a better understanding of the factors that influence endonucleases activity is needed. Indeed, recent studies reported that CRISPR/Cas9-mediated genome editing could influence a p53-mediated DNA damage response.[509, 510] In addition to that, immune responses against bacteria-derived nucleases represent another obstacle to gene editing application.[511]

To overcome these limitations, developments of gene editing tools have multiplied the number of strategies that can be used and as a supplementary example, one can evoke the “safe-harbor locus” approach. Basically, a therapeutic cDNA is inserted in a safe DNA location, i.e. a genomic region where the insertion has no adverse consequences and where the transgene is expressed to achieve therapeutic efficacy. With this objective, different loci were tested in mice such as the Rosa26 and the PPP1R12C loci (also named AAVS1 and known as the integration site of wild-type AAV into the human genome).[407, 408, 512-515] An alternative methodology consists in the insertion of a promoterless construct in the highly expressed albumin locus. In fact, this specific location proposes important advantages including liver specificity and high expression of the therapeutic cDNA. This strategy was explored for different applications, specifically for diseases caused by the deficiency of a secreted protein, such as Hemophilia B for example, but also for other disorders such as OTC, CN syndrome or MPS I.[409, 516-518] Interestingly, insertion in the albumin locus appeared to be more efficient than insertion under the control of the endogenous promoter.[407] Nevertheless, the percentage of edited cells is still quite low (around 1% of total hepatocytes corrected) and insufficient to hope curing most of IEMs. In order to increase the homologous recombination efficiency, recent works used AAV-HSPCs delivery vehicles (a group of

AAVs isolated from human CD34+ HSPCs), and achieved higher efficacy of genome editing.[519] This approach was notably tested in PKU mouse model.[520] Avoiding off-target effects induced by DNA nucleases, these "nuclease-free" strategies help the field in proposing safer gene editing approaches.

Finally, last example of alternative gene editing strategy is the use of an engineered Cas9. This nuclease can be fused to transcriptional activators or repressors in order to transiently modify the gene expression. For instance, a study in which Cas9 was fused to the transcriptional repressor Krüppel-associated box (KRAB) and performed in mouse model of FH, demonstrated encouraging efficacy.[521] A "dead" Cas9, meaning without activity, can also be fused to cytidine or adenosine deaminase, generating nucleotide mismatches.[522, 523] Unfortunately, the increase in size of this kind of engineered nucleases compromises the AAV packaging. For this reason, non-viral delivery methods are not without interest.[524-527]

New development to unlock the potential of AAV-based LGT

With growing preclinical and clinical experience, AAV-based LGT has showed very exciting results and provided crucial knowledge on the approach. This experience represents the key to achieve safe and long-term efficacy for various indications including IEMs. However, a number of obstacles still remain and require further investigation and optimization.[362] The concerns, previously evoked in this manuscript, include the immunogenicity (e.g. pre-existing immunity against AAV vectors, immune response against transduced hepatocytes), a risk of genotoxicity, the absence of persistence in a growing liver or also the risk of acute toxicity at very high doses ($> 10^{14}$ viral genomes/kg). Because of the potential onset of these adverse events over time, it seems obvious to remain cautious and careful monitor the treated patients over a number of years, to evaluate the safety and efficacy of this platform and improve the existing gene therapy tools.

To overcome these current limitations, the research and development work is fundamental. The development of new vectors, more potent, less immunogenic, is necessary to achieve long-term efficacy. The current efforts are centered on the design of novel capsids but also promoters and regulatory elements that outperform the conventional ones, maximizing the transgene expression and allowing the use of safer vector doses **(Figure 1.19)**.[528]

While a single administration of rAAV may be sufficient to achieve a lifelong correction in diseases with low therapeutic threshold (like Hemophilia), vector efficacy and potency are major challenges for the treatment of diseases requiring robust levels of transgene expression. In fact, because the majority of IEMs are cell autonomous, meaning that each hepatocyte is an affected cell that needs to be corrected, they require a high percentage of transduction efficacy to be cured. For example, in disorders like AIP or PH1, it was demonstrated than more than 50% of the hepatocytes need to be corrected. A high vector dose could be used to achieve this level of correction, but recent clinical experiences reported that it increases the risk of treatment-related adverse effects, including CTL responses against transduced hepatocytes.[529] Moreover, the death of three young boys included in the Audentes XLMTM trial demonstrates that there is a dose limit not to be exceeded.

In addition, vector dilution and loss of transgene expression due to liver growth is another major hurdle. This is particularly important for the treatment of IEMs in infancy or childhood or for those diseases in which there is an important level of liver cell proliferation. Transient transgene expression following AAV LGT was documented in a number of studies, including in humans. In particular, in a recent clinical trial for CN syndrome

(NCT03223194), despite obvious evidences of therapeutic efficacy during the first weeks after injection, the disease correction observed in the patient was rapidly lost over time.[530] This loss of transgene expression did not appear to be due to an immune response against the AAV vector and occurred in a timing inconsistent with hepatocyte renewal.[531, 532] Although the intrinsic metabolic impairment associated with the disease in humans was possibly implicated, the mechanisms responsible for the reduced vector persistence are still unclear and vastly unexplored.

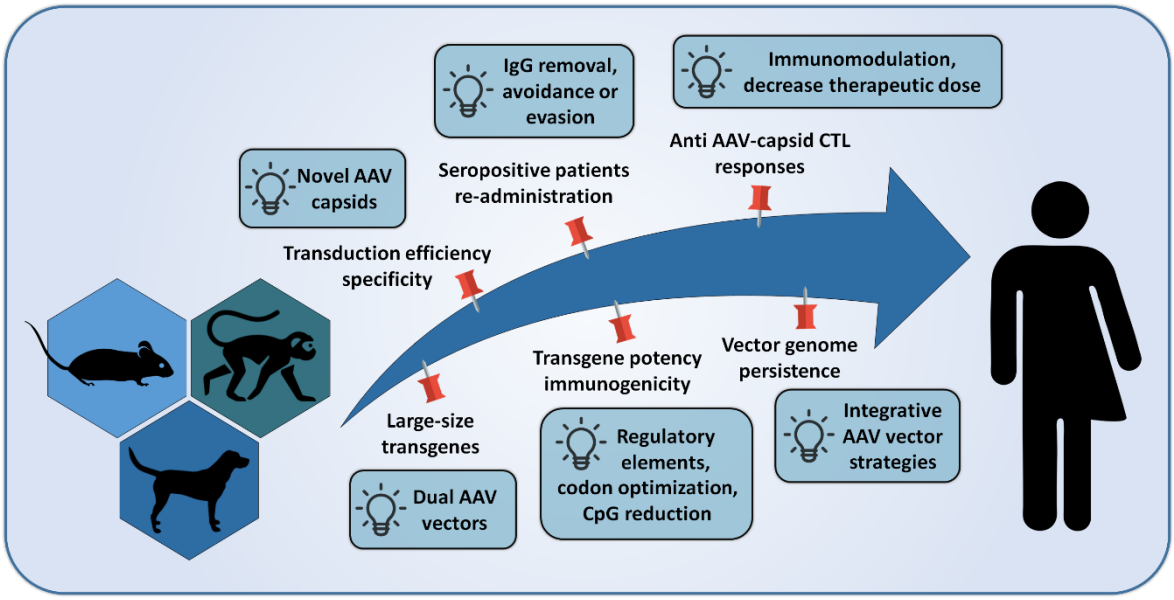


Figure 1.19 Optimization strategies of AAV-based approaches for human gene therapy
Preclinical studies performed in animal models are key to address the current limitations of AAV gene therapy pproaches (red pins) related to the vector genome and the transgene expression cassette (bottom pins) or the vector capsid (upper pins). Examples of various available strategies aimed at overcoming these limitations are depicted (boxes). CTL, cytotoxic T lymphocyte; IgG, immunoglobulin G. Adpated from Colella et al., 2018[362]

An alternative to the loss of transgene expression over time could be the re-administration of the therapeutic vector, which is still today hampered by the immune responses, notably the presence of anti-AAV NAbs. For this reason, the development of strategies to overcome humoral immunity to AAVs is crucial and is the subject of a number of research works.[529, 533-536]

Switching of AAV serotypes could be a first efficient strategy, already shown to be effective in some instances,[465, 537] but is limited by the high degree of cross-reactivity of NAbs across AAV serotypes.[306] To translate this strategy to the clinic, this involves development and production of more than one therapeutic vector, that is not really feasible from an economic point of view and complex from a regulatory point of view. Developing new capsids, engineered to be less susceptible to NAbs and more efficient in transducing their target tissue, is a second option. Nowadays, this R&D field is very active, and frequently, innovative capsids appear in the literature. They are issued from capsid bioengineering, directed mutagenesis, or capsid libraries shuffling strategies.[488, 538-540] In order to avoid detection by the immune system, capsids can also be chemically modified, PEGylated for instance, or associated with exosomes (exo-AAV).[541-543] What can be reproached for these strategies, although efficient, is the reducing of interactions with AAV-specific cell receptors, and so the non-specificity of the transduction. The use of empty capsids to adsorb anti-AAV antibodies, thus allowing for higher transduction rates, was proposed as an

additional option.[317] From a manufacturing point of view, this is an easy strategy to translate to the clinic, since the majority of good manufacturing practices (GMP) AAV vectors batches are purified by affinity column, where empty and full rAAV particles are not separated. However, the presence of these empty capsids increases considerably the antigen load in the target organ and may potentially trigger anti capsid T-cell immunity.[544]

On the other hand, the most effective strategies so far were based on the pharmacological and physical modulations of anti-AAV NAbs. A recurrent, straightforward and effective way to prevent or eradicate humoral immune responses to AAV is the use of immunosuppressive drugs. Certain treatments are already approved for use in humans, notably those that selectively target B cells and plasma cells (e.g. rituximab, bortezomib, anti-CD4, sirolimus, tacrolimus, etc.) These drugs are used to specifically deplete an immune cell population or to prevent the generation of antibodies and the antigen presentation.[545] Unfortunately, immunosuppression come with certain downsides for the patient. Indeed, with this kind of treatment administered for a certain amount of time, usually, several weeks, we expose the patients to higher risk of pathogen infections. The recent development of tolerogenic nanoparticles containing rapamycin (ImmTOR®, Selecta Biosciences Inc.) seems to be interesting and promising. Indeed, several works reported efficient and specific modulation of AAV immunogenicity, enabling vector re-administration.[546, 547] Another option to overcome pre-existing antibodies is the use of an endopeptidase from bacteria, that is able to degrade circulating IgG, called Imlifidase (IdeS). This enzyme is currently being tested in transplantation and could be also a potential solution in AAV gene therapy.[548] Very recent works demonstrate that IdeS administration decreased anti-AAV antibodies and enabled efficient LGT in NHP.[549] A last example of strategy to overcome humoral immunity seropositive to AAVs, is the physical removal of antibodies. Plasmapheresis and immuno-adsorption belongs to this category. They are already used in the clinic for autoimmune diseases caused by high auto-antibody levels (e.g. Guillain-Barré or myasthenia gravis) and were successfully applied in animal models for AAV gene therapy.[550, 551]. Recently, an AAV-specific plasmapheresis column has been developed, which was shown to efficiently and selectively deplete anti-AAV antibodies without depleting the total immunoglobulin pool from plasma.[552]

To reduce the impact of the T-cell immune response directed against the AAV capsid, transaminases level has been carefully monitored and steroids administered in subjects enrolled in clinical trials. However, to date, there is still no consensus on the optimal protocol for the use of steroids to apply (e.g. timing of intervention, dose, duration, co-treatment with other immunosuppressive drugs, etc...) and the most effective immunosuppressive strategy is still debated.[280] Different factors, directly linked to the vector, influence the CTL response against AAV-transduced cells, such as the vector dose, the DNA conformation, the presence of CpG dinucleotides or the amount of empty capsids or impurities, etc.[305, 311, 529] Scientists have to take into account these factors in the development of the next generation of vectors (**Figure 1.19**).

More broadly, there are other major obstacles to the development of innovative AAV-based gene therapy drugs. The bioproduction at the industrial scale is one of them. The manufacturing processes are still for a large part derived from academic research and are not always suitable for large-scale deployment in accordance with the GMP applied in pharmaceutical production. Production yields have to be improved and for that, technological and industrial breakthrough innovations have to be rapidly developed. This is particularly relevant regarding the doses that are needed to treat a patient suffering from a disease affecting the entire body. The cost of gene therapy drugs is another issue : Glybera costs around one million euros, Strimvelis over 600,000€, 320,000€ are asked for Kymriah,

Luxturna is fixed around 850,000€ for both eyes treatment, and finally Zolgensma has been announced at 1.9 million euros, being the most expensive treatment in the world per unit. Of course, these prices take account of development and production costs, and could be justified by their therapeutic value and the resulting reduction in the cost of ongoing treatments administered to people with rare genetic diseases. However, at these prices, how to ensure that all patients will have an access to the gene therapy drugs?

2.3 Concluding remarks

Nowadays, we look forward to the time when improved diagnosis and available treatments will make morbidity and mortality in IEMs things of the past. We are not here yet, but real progresses were made during the last decades. These advances also bring challenges for medicine and society. As more and more complex metabolic mechanisms are understood, the very nature of IEMs continues to evolve. This is specially true as we explore the emerging role of epigenetics and we start to understand metabolic disturbances caused by cancers, immune dysfunction, ageing, and other disorders. New therapeutic options are available for certain IEMs, but are generally very expensive. Not all insurance programs cover these drugs or non-prescription supplements needed for some disorders. Thus, screening these diseases, without the guarantee of providing affordable treatment to all affected people, is a real ethical and economic challenge.

There are many opportunities to discover new treatments for IEMs. Finding solutions for the most pressing medical needs is motivating and exciting. Gene therapy has the potential to transform patients' lives. In this field, great advances have been made so far, highlighted by the approval of several drugs and promising ongoing clinical trials. Major progresses have been made possible thanks to the common efforts of scientists, clinicians, patient associations, biotechs and pharmaceutical companies. In particular, several patient associations have been committed for many years to the development of gene therapy through their financial support of research. In parallel, more and more industrial stakeholders are working hard to establish an advanced drugs production sector. Gene therapy and genome editing medicines are among the most complex drugs developed up to now. The long-lasting therapeutic benefits they bring to certain IEMs are beginning to appear and justify continuing efforts to treat other currently incurable genetic disorders in the near future. We are learning from each clinical trial and expanding our knowledge on both therapeutic efficacy and drawbacks of gene therapy vectors in humans. Important issues, from genotoxicity to the immune responses and higher and persistent efficiency, remain to be addressed. Moreover, a variety of strategies has to be developed to counteract specific IEMs biological aspects, such as early onset or multi-systemic involvement.

3. Scope of the thesis

The overall goal of the works carried out during this thesis was to develop and support clinical application of advanced therapies for rare IEMs. In particular, efforts were focused on design, optimization and preclinical development of innovative gene therapy approaches to treat PD and CN syndrome. Based on the current knowledge on these diseases and therapeutic strategies attempted so far, we developed different approaches in the purpose of providing patients with access to the most advanced research biologics.

These translational research works will be presented in two parts: the first will focus especially in PD and it will scope out potential new treatments based on AAV-mediated gene

therapy. After an overview of the therapeutic options and the challenges facing PD (**Chapter 2**), the results that will be reported will cover substrate reduction strategy (**Chapter 3**), as well as the design of novel AAV vectors to improve muscle targeting (**Chapter 4**). Investigations of complementary approaches to improve AAV-based LGT will also be discussed (**Chapter 5**).

The second part of the thesis is dedicated to CN syndrome (**Chapter 6**) and how we translated proof of concept studies to humans (**Chapter 7**). Works of early and late phase of development will be presented in this chapter including the preclinical development of an AAV-based LGT. These studies have resulted in a clinical trial, which is currently ongoing. The works that support this trial, notably addressing immunomodulation evaluation will also be presented in this manuscript.

Finally, the last chapter will summarize the other works performed during the PhD and will conlude the thesis (**Chapter 8**).

Through all the different studies of diverse stage of development that will be presented there, this thesis illustrates the long journey of a drug candidate from the lab bench to arrive at the bedside of patients (**Figure 1.20**).

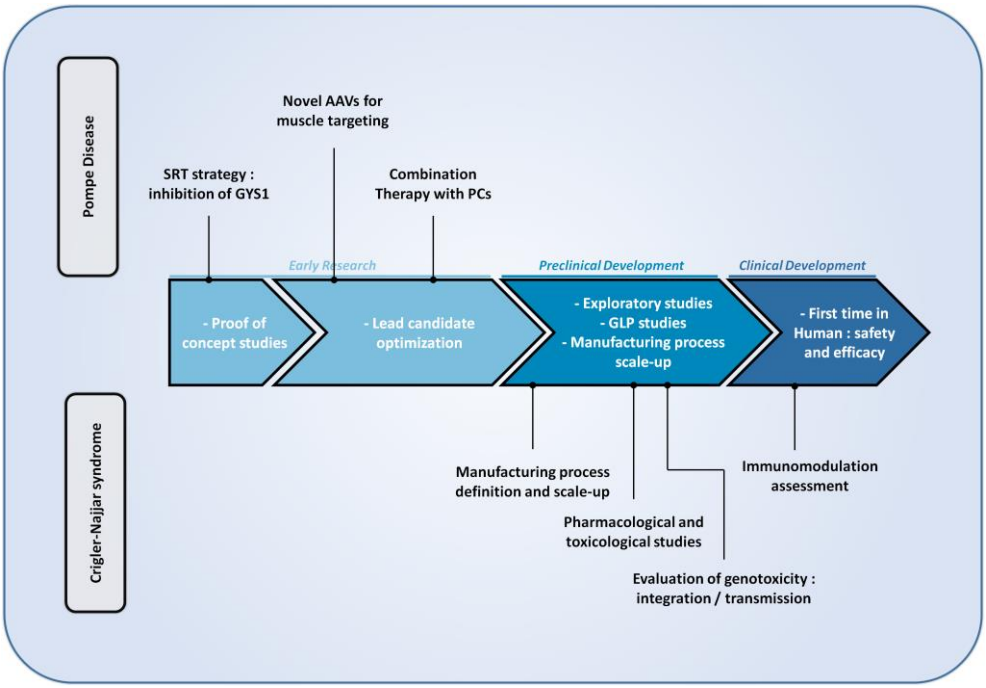


Figure 1.20 Studies performed during the thesis and their location on a drug development scale

4. References

1. Garrod, A.E. About Alkaptonuria. *Med Chir Trans* **85**, 69-78 (1902).
2. Scriver, C.R. Garrod's Croonian Lectures (1908) and the charter 'Inborn Errors of Metabolism': albinism, alkaptonuria, cystinuria, and pentosuria at age 100 in 2008. *J Inherit Metab Dis* **31**, 580-98 (2008).
3. Følling, I. The discovery of phenylketonuria. *Acta Paediatrica* **83**, 4-10 (1994).
4. Krebs, H.A. The citric acid cycle: A reply to the criticisms of FL Breusch and of J. Thomas. *Biochemical Journal* **34**, 460 (1940).
5. Krebs, H. Urea formation in mammalian liver. *Biochemical Journal* **36**, 758 (1942).

6. Goodman, S.I. & Markey, S.P. Diagnosis of organic acidemias by gas chromatography-mass spectrometry (AR Liss, 1981).
7. Morava, E. et al. Quo vadis: the re-definition of "inborn metabolic diseases". *J Inherit Metab Dis* **38**, 1003-6 (2015).
8. Ferreira, C.R., van Karnebeek, C.D.M., Vockley, J. & Blau, N. A proposed nosology of inborn errors of metabolism. *Genet Med* **21**, 102-106 (2019).
9. Campeau, P.M., Scriver, C.R. & Mitchell, J.J. A 25-year longitudinal analysis of treatment efficacy in inborn errors of metabolism. *Mol Genet Metab* **95**, 11-6 (2008).
10. Waters, D. et al. Global birth prevalence and mortality from inborn errors of metabolism: a systematic analysis of the evidence. *J Glob Health* **8**, 021102 (2018).
11. McCandless, S.E., Brunger, J.W. & Cassidy, S.B. The burden of genetic disease on inpatient care in a children's hospital. *The American Journal of Human Genetics* **74**, 121-127 (2004).
12. Scriver, C.R. et al. The Metabolic and Molecular Bases of Inherited Disease (2001).
13. Alfadhel, M. et al. Thirteen year retrospective review of the spectrum of inborn errors of metabolism presenting in a tertiary center in Saudi Arabia. *Orphanet J Rare Dis* **11**, 126 (2016).
14. Arnold, G.L. Inborn errors of metabolism in the 21st century: past to present. *Annals of translational medicine* **6** (2018).
15. Saudubray, J.M. & Garcia-Cazorla, A. Inborn Errors of Metabolism Overview: Pathophysiology, Manifestations, Evaluation, and Management. *Pediatr Clin North Am* **65**, 179-208 (2018).
16. Saudubray, J.M., Baumgartner, M. & Walter, J. Inborn Metabolic Diseases, Diagnosis and Treatment (ed. 6th) (Springer, 2016).
17. Agana, M., Frueh, J., Kamboj, M., Patel, D.R. & Kanungo, S. Common metabolic disorder (inborn errors of metabolism) concerns in primary care practice. *Ann Transl Med* **6**, 469 (2018).
18. Vernon, H.J. Inborn Errors of Metabolism: Advances in Diagnosis and Therapy. *JAMA Pediatr* **169**, 778-82 (2015).
19. Therrell, B.L. et al. Current status of newborn screening worldwide: 2015. *Semin Perinatol* **39**, 171-87 (2015).
20. Fabie, N.A.V., Pappas, K.B. & Feldman, G.L. The Current State of Newborn Screening in the United States. *Pediatr Clin North Am* **66**, 369-386 (2019).
21. Scott, C.R. et al. Newborn Screening for Mucopolysaccharidoses: Results of a Pilot Study with 100 000 Dried Blood Spots. *J Pediatr* **216**, 204-207 (2020).
22. Hong, X., Sadilek, M. & Gelb, M.H. A highly multiplexed biochemical assay for analytes in dried blood spots: application to newborn screening and diagnosis of lysosomal storage disorders and other inborn errors of metabolism. *Genet Med* (2020).
23. Wilson, J.M.G., Jungner, G. & Organization, W.H. Principles and practice of screening for disease. (1968).
24. Health Resources and Services Administration. Advisory Committee on Heritable Disorders in Newborns and Children: Recommended Uniform Screenind Panel (Secretary of the Department of Health and Human Services, https://www.hrsa.gov/advisory-committees/heritable-disorders/rusp/index.html, 2020).
25. Rice, G.M. & Steiner, R.D. Inborn Errors of Metabolism (Metabolic Disorders). *Pediatr Rev* **37**, 3-15; quiz 16-7, 47 (2016).
26. Lee, J.J.Y., Wasserman, W.W., Hoffmann, G.F., van Karnebeek, C.D.M. & Blau, N. Knowledge base and mini-expert platform for the diagnosis of inborn errors of metabolism. *Genet Med* **20**, 151-158 (2018).
27. Alfadhel, M., Al-Thihli, K., Moubayed, H., Eyaid, W. & Al-Jeraisy, M. Drug treatment of inborn errors of metabolism: a systematic review. *Archives of disease in childhood* **98**, 454-461 (2013).
28. Gambello, M.J. & Li, H. Current strategies for the treatment of inborn errors of metabolism. *J Genet Genomics* **45**, 61-70 (2018).
29. Ginocchio, V.M. & Brunetti-Pierri, N. Progress toward improved therapies for inborn errors of metabolism. *Hum Mol Genet* **25**, R27-35 (2016).
30. Schwartz, I.V.D., Souza, C.F.M.d. & Giugliani, R. Treatment of inborn errors of metabolism. *Jornal de pediatria. Porto Alegre. Vol. 84, n. 4 supl.(jul./ago. 2008), p. S8-S19* (2008).
31. Berry, S.A. et al. Medical Foods for Inborn Errors of Metabolism: History, Current Status, and Critical Need. *Pediatrics* **145** (2020).
32. Jameson, E. & Morris, A. Nutrition in metabolic disease. *Paediatrics and Child Health* **21**, 401-405 (2011).
33. McCorvie, T.J. & Timson, D.J. in Protein Homeostasis Diseases 221-245 (Elsevier, 2020).
34. Collins, J. Metabolic disease. Time for fructose solutions to go. *Lancet (London, England)* **341**, 600-600 (1993).
35. Schouwink, G. De hepatocerebrale degeneratie, met een onderzoek naar de koperstofwisseling. *University of Amsterdam MD thesis* (1961).

36. Brewer, G.J. et al. Treatment of Wilson's disease with zinc: XV long-term follow-up studies. *Journal of Laboratory and Clinical Medicine* **132**, 264-278 (1998).
37. Camarata, M.A., Ala, A. & Schilsky, M.L. Zinc maintenance therapy for Wilson disease: a comparison between zinc acetate and alternative zinc preparations. *Hepatology communications* **3**, 1151-1158 (2019).
38. Singh, R.H. et al. Updated, web-based nutrition management guideline for PKU: An evidence and consensus based approach. *Mol Genet Metab* **118**, 72-83 (2016).
39. Sentner, C.P. et al. Glycogen storage disease type III: diagnosis, genotype, management, clinical course and outcome. *J Inherit Metab Dis* **39**, 697-704 (2016).
40. Derks, T.G. & Smit, G.P. Dietary management in glycogen storage disease type III: what is the evidence? *J Inherit Metab Dis* **38**, 545-50 (2015).
41. Klepper, J. et al. Introduction of a ketogenic diet in young infants. *Journal of inherited metabolic disease* **25**, 449-460 (2002).
42. Nassogne, M.-C., Heron, B., Touati, G., Rabier, D. & Saudubray, J. Urea cycle defects: management and outcome. *Journal of inherited metabolic disease* **28**, 407-414 (2005).
43. Wolf, B. Biotinidase deficiency:"if you have to have an inherited metabolic disease, this is the one to have". *Genetics in medicine* **14**, 565-575 (2012).
44. Wolf, B. Successful outcomes of older adolescents and adults with profound biotinidase deficiency identified by newborn screening. *Genetics in Medicine* **19**, 396-402 (2017).
45. Stöckler, S., Hanefeld, F. & Frahm, J. Creatine replacement therapy in guanidineoacetate methyltransferase deficiency, a novel inborn error of metabolism. *The Lancet* **348**, 789-790 (1996).
46. Baumgartner, M.R. Vitamin-responsive disorders: cobalamin, folate, biotin, vitamins B1 and E. *Handb Clin Neurol* **113**, 1799-810 (2013).
47. Rodrigues, J.V., Henriques, B.J., Lucas, T.G. & Gomes, C.M. Cofactors and metabolites as protein folding helpers in metabolic diseases. *Curr Top Med Chem* **12**, 2546-59 (2012).
48. Boyer, S.W., Barclay, L.J. & Burrage, L.C. Inherited Metabolic Disorders: Aspects of Chronic Nutrition Management. *Nutr Clin Pract* **30**, 502-10 (2015).
49. Concolino, D., Deodato, F. & Parini, R. Enzyme replacement therapy: efficacy and limitations. *Italian journal of pediatrics* **44**, 120 (2018).
50. Fratantoni, J.C., Hall, C.W. & Neufeld, E.F. Hurler and Hunter syndromes: mutual correction of the defect in cultured fibroblasts. *Science* **162**, 570-2 (1968).
51. Dahms, N.M., Lobel, P. & Kornfeld, S. Mannose 6-phosphate receptors and lysosomal enzyme targeting. *J Biol Chem* **264**, 12115-8 (1989).
52. Coutinho, M.F., Prata, M.J. & Alves, S. Mannose-6-phosphate pathway: a review on its role in lysosomal function and dysfunction. *Molecular genetics and metabolism* **105**, 542-550 (2012).
53. Parenti, G., Pignata, C., Vajro, P. & Salerno, M. New strategies for the treatment of lysosomal storage diseases. *International journal of molecular medicine* **31**, 11-20 (2012).
54. Wyatt, K. et al. The effectiveness and cost-effectiveness of enzyme and substrate replacement therapies: a longitudinal cohort study of people with lysosomal storage disorders. (2012).
55. Lachmann, R.H. Enzyme replacement therapy for lysosomal storage diseases. *Current opinion in pediatrics* **23**, 588-593 (2011).
56. Ebbink, B.J. et al. Cognitive decline in classic infantile Pompe disease: An underacknowledged challenge. *Neurology* **86**, 1260-1261 (2016).
57. Pardridge, W.M. The blood-brain barrier: bottleneck in brain drug development. *NeuroRx* **2**, 3-14 (2005).
58. Fecarotta, S., Gasperini, S. & Parenti, G. New treatments for the mucopolysaccharidoses: from pathophysiology to therapy. *Italian journal of pediatrics* **44**, 135-143 (2018).
59. Kishnani, P.S. et al. Cross-reactive immunologic material status affects treatment outcomes in Pompe disease infants. *Mol Genet Metab* **99**, 26-33 (2010).
60. Desai, A.K., Kazi, Z.B., Bali, D.S. & Kishnani, P.S. Characterization of immune response in Cross-Reactive Immunological Material (CRIM)-positive infantile Pompe disease patients treated with enzyme replacement therapy. *Molecular genetics and metabolism reports* **20**, 100475 (2019).
61. Giugliani, R. et al. Immune tolerance induction for laronidase treatment in mucopolysaccharidosis I. *Molecular genetics and metabolism reports* **10**, 61-66 (2017).
62. Tan, E.Y., Boelens, J.J., Jones, S.A. & Wynn, R.F. Haematopoietic Stem Cell Transplantation in Inborn Errors of Metabolism-On Behalf of IEWP-EBMT. *Frontiers in pediatrics* **7**, 433 (2019).
63. Chiesa, R., Wynn, R.F. & Veys, P. Haematopoietic stem cell transplantation in inborn errors of metabolism. *Current opinion in hematology* **23**, 530-535 (2016).
64. Hadzic, N. & Vara, R. in Pediatric Hepatology and Liver Transplantation 271-278 (Springer, 2019).
65. D'Antiga, L. Pediatric Hepatology and Liver Transplantation (Springer, 2019).
66. Fagiuoli, S., Daina, E., D'Antiga, L., Colledan, M. & Remuzzi, G. Monogenic diseases that can be cured by liver transplantation. *Journal of hepatology* **59**, 595-612 (2013).

67. Burlina, A. & D'Antiga, L. in Pediatric Hepatology and Liver Transplantation 603-624 (Springer, 2019).
68. Mazariegos, G. et al. Liver transplantation for pediatric metabolic disease. *Molecular genetics and metabolism* **111**, 418-427 (2014).
69. Ciria, R., Davila, D. & Heaton, N. Auxiliary liver transplantation in children. *Current opinion in organ transplantation* **16**, 489-493 (2011).
70. Popescu, I. & Dima, S.O. Domino liver transplantation: how far can we push the paradigm? *Liver Transplantation* **18**, 22-28 (2012).
71. Oishi, K., Arnon, R., Wasserstein, M.P. & Diaz, G.A. Liver transplantation for pediatric inherited metabolic disorders: considerations for indications, complications, and perioperative management. *Pediatric transplantation* **20**, 756-769 (2016).
72. Wagener, G. Multiple listings: Good for a few, but no solution for the organ shortage. *Transplantation* **104**, 671-672 (2020).
73. Janardhan, S. et al. Recent advances in the development of pharmaceutical agents for metabolic disorders: A computational perspective. *Current medicinal chemistry* **25**, 5432-5463 (2018).
74. Matalonga, L., Gort, L. & Ribes, A. Small molecules as therapeutic agents for inborn errors of metabolism. *Journal of inherited metabolic disease* **40**, 177-193 (2017).
75. Schreiber, S.L. et al. Advancing biological understanding and therapeutics discovery with small-molecule probes. *Cell* **161**, 1252-1265 (2015).
76. Balchin, D., Hayer-Hartl, M. & Hartl, F.U. In vivo aspects of protein folding and quality control. *Science* **353**, aac4354 (2016).
77. Morello, J.-P. et al. Pharmacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. *The Journal of clinical investigation* **105**, 887-895 (2000).
78. Ringe, D. & Petsko, G.A. Q&A: What are pharmacological chaperones and why are they interesting? *Journal of biology* **8**, 80 (2009).
79. Parenti, G., Andria, G. & Valenzano, K.J. Pharmacological chaperone therapy: preclinical development, clinical translation, and prospects for the treatment of lysosomal storage disorders. *Molecular Therapy* **23**, 1138-1148 (2015).
80. Liguori, L. et al. Pharmacological Chaperones: A Therapeutic Approach for Diseases Caused by Destabilizing Missense Mutations. *International Journal of Molecular Sciences* **21**, 489 (2020).
81. Hou, Z.-S., Ulloa-Aguirre, A. & Tao, Y.-X. Pharmacoperone drugs: targeting misfolded proteins causing lysosomal storage-, ion channels-, and G protein-coupled receptors-associated conformational disorders. *Expert review of clinical pharmacology* **11**, 611-624 (2018).
82. Pereira, D.M., Valentão, P. & Andrade, P.B. Tuning protein folding in lysosomal storage diseases: the chemistry behind pharmacological chaperones. *Chemical science* **9**, 1740-1752 (2018).
83. Tao, Y.-X. & Conn, P.M. Pharmacoperones as novel therapeutics for diverse protein conformational diseases. *Physiological reviews* **98**, 697-725 (2018).
84. Muntau, A.C., Leandro, J., Staudigl, M., Mayer, F. & Gersting, S.W. Innovative strategies to treat protein misfolding in inborn errors of metabolism: pharmacological chaperones and proteostasis regulators. *Journal of inherited metabolic disease* **37**, 505-523 (2014).
85. Parenti, G. Treating lysosomal storage diseases with pharmacological chaperones: from concept to clinics. *EMBO molecular medicine* **1**, 268-279 (2009).
86. Benito, J.M., García Fernández, J.M. & Mellet, C.O. Pharmacological chaperone therapy for Gaucher disease: a patent review. *Expert opinion on therapeutic patents* **21**, 885-903 (2011).
87. Kishnani, P. et al. First-in-human study of advanced and targeted acid α-glucosidase (AT-GAA)(ATB200/AT2221) in patients with Pompe disease: preliminary functional assessment results from the ATB200-02 trial. *Molecular Genetics and Metabolism* **126**, S86 (2019).
88. Khanna, R. et al. The pharmacological chaperone AT2220 increases the specific activity and lysosomal delivery of mutant acid alpha-glucosidase, and promotes glycogen reduction in a transgenic mouse model of Pompe disease. *PLoS One* **9**, e102092 (2014).
89. Parenti, G. et al. A chaperone enhances blood alpha-glucosidase activity in Pompe disease patients treated with enzyme replacement therapy. *Mol Ther* **22**, 2004-12 (2014).
90. Germain, D.P. et al. Treatment of Fabry's disease with the pharmacologic chaperone migalastat. *New England Journal of Medicine* **375**, 545-555 (2016).
91. Ortiz, A. et al. Fabry disease revisited: management and treatment recommendations for adult patients. *Molecular genetics and metabolism* **123**, 416-427 (2018).
92. Amicus Therapeutics. Galafold™ (migalastat) capsules, for oral use: US prescribing information (https://www.fda.gov/, 2018).
93. European Medicines Agency. Migalastat (Galafold): EU summary of product characteristics. (https://www.ema.europa.eu/, 2018).

94. Clancy, J. et al. Results of a phase IIa study of VX-809, an investigational CFTR corrector compound, in subjects with cystic fibrosis homozygous for the F508del-CFTR mutation. *Thorax* **67**, 12-18 (2012).
95. Wainwright, C.E. et al. Lumacaftor–ivacaftor in patients with cystic fibrosis homozygous for Phe508del CFTR. *New England Journal of Medicine* **373**, 220-231 (2015).
96. Sanford, M. & Keating, G.M. Sapropterin. *Drugs* **69**, 461-476 (2009).
97. Pey, A.L. et al. Mechanisms underlying responsiveness to tetrahydrobiopterin in mild phenylketonuria mutations. *Human mutation* **24**, 388-399 (2004).
98. Evers, R.A., van Vliet, D. & van Spronsen, F.J. Tetrahydrobiopterin treatment in phenylketonuria: A repurposing approach. *Journal of inherited metabolic disease* **43**, 189-199 (2020).
99. Cosentino, F. et al. Chronic treatment with tetrahydrobiopterin reverses endothelial dysfunction and oxidative stress in hypercholesterolaemia. *Heart* **94**, 487-492 (2008).
100. Heitzer, T., Krohn, K., Albers, S. & Meinertz, T. Tetrahydrobiopterin improves endothelium-dependent vasodilation by increasing nitric oxide activity in patients with Type II diabetes mellitus. *Diabetologia* **43**, 1435-1438 (2000).
101. Abudukadier, A. et al. Tetrahydrobiopterin has a glucose-lowering effect by suppressing hepatic gluconeogenesis in an endothelial nitric oxide synthase–dependent manner in diabetic mice. *Diabetes* **62**, 3033-3043 (2013).
102. Porkert, M. et al. Tetrahydrobiopterin: a novel antihypertensive therapy. *Journal of human hypertension* **22**, 401-407 (2008).
103. Kim, H.K. & Han, J. Tetrahydrobiopterin in energy metabolism and metabolic diseases. *Pharmacological Research*, 104827 (2020).
104. Aflaki, E. et al. A new glucocerebrosidase chaperone reduces α-synuclein and glycolipid levels in iPSC-derived dopaminergic neurons from patients with Gaucher disease and parkinsonism. *Journal of Neuroscience* **36**, 7441-7452 (2016).
105. Porto, C. et al. Pharmacological enhancement of α-glucosidase by the allosteric chaperone N-acetylcysteine. *Molecular Therapy* **20**, 2201-2211 (2012).
106. Bulawa, C.E. et al. Tafamidis, a potent and selective transthyretin kinetic stabilizer that inhibits the amyloid cascade. *Proceedings of the National Academy of Sciences* **109**, 9629-9634 (2012).
107. Generoso, S.F. et al. Pharmacological folding chaperones act as allosteric ligands of Frizzled4. *Nature chemical biology* **11**, 280 (2015).
108. Valenzano, K.J. et al. Identification and characterization of pharmacological chaperones to correct enzyme deficiencies in lysosomal storage disorders. *Assay and drug development technologies* **9**, 213-235 (2011).
109. Flanagan, J.J. et al. The pharmacological chaperone 1-deoxynojirimycin increases the activity and lysosomal trafficking of multiple mutant forms of acid alpha-glucosidase. *Human mutation* **30**, 1683-1692 (2009).
110. Centre For Genomic Regulation. FoldX Suite (http://foldxsuite.crg.eu/).
111. Schrödinger. PyMOL (https://pymol.org/2/).
112. Wu, X. et al. A pharmacogenetic approach to identify mutant forms of α-galactosidase a that respond to a pharmacological chaperone for Fabry disease. *Human mutation* **32**, 965-977 (2011).
113. Lukas, J. et al. In vitro enzyme measurement to test pharmacological chaperone responsiveness in Fabry and Pompe disease. *JoVE (Journal of Visualized Experiments)*, e56550 (2017).
114. Wang, Y.-J., Di, X.-J. & Mu, T.-W. Using pharmacological chaperones to restore proteostasis. *Pharmacological research* **83**, 3-9 (2014).
115. Chambers, J.E. & Marciniak, S.J. Cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. 2. Protein misfolding and ER stress. *American Journal of Physiology-Cell Physiology* **307**, C657-C670 (2014).
116. Hartl, F.U., Bracher, A. & Hayer-Hartl, M. Molecular chaperones in protein folding and proteostasis. *Nature* **475**, 324-332 (2011).
117. Mardones, P., Martínez, G. & Hetz, C. Control of systemic proteostasis by the nervous system. *Trends in cell biology* **25**, 1-10 (2015).
118. Balch, W.E., Morimoto, R.I., Dillin, A. & Kelly, J.W. Adapting proteostasis for disease intervention. *science* **319**, 916-919 (2008).
119. Lee, B.S., Chen, J., Angelidis, C., Jurivich, D.A. & Morimoto, R.I. Pharmacological modulation of heat shock factor 1 by antiinflammatory drugs results in protection against stress-induced cellular damage. *Proceedings of the National Academy of Sciences* **92**, 7207-7211 (1995).
120. Westerheide, S.D. et al. Celastrols as inducers of the heat shock response and cytoprotection. *Journal of Biological Chemistry* **279**, 56053-56060 (2004).
121. Trott, A. et al. Activation of heat shock and antioxidant responses by the natural product celastrol: transcriptional signatures of a thiol-targeted molecule. *Molecular biology of the cell* **19**, 1104-1112 (2008).

122. Vaughan, C.K., Neckers, L. & Piper, P.W. Understanding of the Hsp90 molecular chaperone reaches new heights. *Nature structural & molecular biology* **17**, 1400-1404 (2010).
123. Neef, D.W., Turski, M.L. & Thiele, D.J. Modulation of heat shock transcription factor 1 as a therapeutic target for small molecule intervention in neurodegenerative disease. *PLoS biology* **8** (2010).
124. Neef, D.W., Jaeger, A.M. & Thiele, D.J. Heat shock transcription factor 1 as a therapeutic target in neurodegenerative diseases. *Nature reviews Drug discovery* **10**, 930-944 (2011).
125. Singh, L.R., Gupta, S., Honig, N.H., Kraus, J.P. & Kruger, W.D. Activation of mutant enzyme function in vivo by proteasome inhibitors and treatments that induce Hsp70. *PLoS genetics* **6** (2010).
126. Westerheide, S.D. & Morimoto, R.I. Heat shock response modulators as therapeutic tools for diseases of protein conformation. *Journal of Biological Chemistry* **280**, 33097-33100 (2005).
127. Mu, T.-W. et al. Chemical and biological approaches synergize to ameliorate protein-folding diseases. *Cell* **134**, 769-781 (2008).
128. Macías-Vidal, J. et al. The proteasome inhibitor bortezomib reduced cholesterol accumulation in fibroblasts from Niemann–Pick type C patients carrying missense mutations. *The FEBS journal* **281**, 4450-4466 (2014).
129. Kisselev, A.F. & Goldberg, A.L. Proteasome inhibitors: from research tools to drug candidates. *Chemistry & Biology* **8**, 739-758 (2001).
130. Wang, F., Chou, A. & Segatori, L. Lacidipine remodels protein folding and Ca2+ homeostasis in Gaucher's disease fibroblasts: a mechanism to rescue mutant glucocerebrosidase. *Chemistry & biology* **18**, 766-776 (2011).
131. Norez, C. et al. Roscovitine is a proteostasis regulator that corrects the trafficking defect of F 508del-CFTR by a CDK-independent mechanism. *British journal of pharmacology* **171**, 4831-4849 (2014).
132. Egan, M.E. et al. Calcium-pump inhibitors induce functional surface expression of ΔF508-CFTR protein in cystic fibrosis epithelial cells. *Nature medicine* **8**, 485-492 (2002).
133. Chen, C.-C. et al. A small molecule restores function to TRPML1 mutant isoforms responsible for mucolipidosis type IV. *Nature communications* **5**, 1-10 (2014).
134. Egan, M.E. et al. Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. *Science* **304**, 600-602 (2004).
135. Lloyd-Evans, E. et al. Niemann-Pick disease type C1 is a sphingosine storage disease that causes deregulation of lysosomal calcium. *Nature medicine* **14**, 1247 (2008).
136. Galluzzi, L. et al. Molecular definitions of autophagy and related processes. *The EMBO journal* **36**, 1811-1836 (2017).
137. Settembre, C. et al. TFEB links autophagy to lysosomal biogenesis. *science* **332**, 1429-1433 (2011).
138. Kim, D.-H. et al. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110**, 163-175 (2002).
139. Puertollano, R. mTOR and lysosome regulation. *F1000prime reports* **6** (2014).
140. Rabanal-Ruiz, Y., Otten, Elsje G. & Korolchuk, Viktor I. mTORC1 as the main gateway to autophagy. *Essays in Biochemistry* **61**, 565-584 (2017).
141. Ballabio, A. & Gieselmann, V. Lysosomal disorders: from storage to cellular damage. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* **1793**, 684-696 (2009).
142. Lieberman, A.P. et al. Autophagy in lysosomal storage disorders. *Autophagy* **8**, 719-730 (2012).
143. Platt, F.M., Boland, B. & van der Spoel, A.C. Lysosomal storage disorders: The cellular impact of lysosomal dysfunction. *Journal of Cell Biology* **199**, 723-734 (2012).
144. Rubinsztein, D.C., Codogno, P. & Levine, B. Autophagy modulation as a potential therapeutic target for diverse diseases. *Nature reviews Drug discovery* **11**, 709-730 (2012).
145. Schneider, J.L. & Cuervo, A.M. Autophagy and human disease: emerging themes. *Current opinion in genetics & development* **26**, 16-23 (2014).
146. Spampanato, C. et al. Transcription factor EB (TFEB) is a new therapeutic target for Pompe disease. *EMBO molecular medicine* **5**, 691-706 (2013).
147. Rega, L.R. et al. Activation of the transcription factor EB rescues lysosomal abnormalities in cystinotic kidney cells. *Kidney international* **89**, 862-873 (2016).
148. Moskot, M. et al. The phytoestrogen genistein modulates lysosomal metabolism and transcription factor EB (TFEB) activation. *Journal of Biological Chemistry* **289**, 17054-17069 (2014).
149. Tamura, A. & Yui, N. β-Cyclodextrin-threaded biocleavable polyrotaxanes ameliorate impaired autophagic flux in Niemann-Pick type C disease. *Journal of Biological Chemistry* **290**, 9442-9454 (2015).
150. Maarup, T.J. et al. Intrathecal 2-hydroxypropyl-beta-cyclodextrin in a single patient with Niemann–Pick C1. *Molecular genetics and metabolism* **116**, 75-79 (2015).
151. García-Robles, A.A. et al. Use of 2 hydroxypropyl-beta-cyclodextrin therapy in two adult Niemann Pick Type C patients. *Journal of the neurological sciences* **366**, 65 (2016).

152. Berry-Kravis, E. et al. Long-term treatment of Niemann-Pick type C1 disease with intrathecal 2-hydroxypropyl-β-cyclodextrin. *Pediatric neurology* **80**, 24-34 (2018).
153. Zhang, S. et al. Evaluation of autophagy inducers in epithelial cells carrying the ΔF508 mutation of the cystic fibrosis transmembrane conductance regulator CFTR. *Cell death & disease* **9**, 1-12 (2018).
154. Stefano, D.D. et al. Restoration of CFTR function in patients with cystic fibrosis carrying the F508del-CFTR mutation. *Autophagy* **10**, 2053-2074 (2014).
155. Johnson, S.C. et al. mTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome. *Science* **342**, 1524-1528 (2013).
156. Kuo, S.-Y. et al. Small-molecule enhancers of autophagy modulate cellular disease phenotypes suggested by human genetics. *Proceedings of the National Academy of Sciences* **112**, E4281-E4287 (2015).
157. Sarkar, S. Regulation of autophagy by mTOR-dependent and mTOR-independent pathways: autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers. *Biochemical Society Transactions* **41**, 1103-1130 (2013).
158. Bidou, L., Allamand, V., Rousset, J.-P. & Namy, O. Sense from nonsense: therapies for premature stop codon diseases. *Trends in molecular medicine* **18**, 679-688 (2012).
159. Keeling, K.M., Wang, D., Conard, S.E. & Bedwell, D.M. Suppression of premature termination codons as a therapeutic approach. *Critical reviews in biochemistry and molecular biology* **47**, 444-463 (2012).
160. Shalev, M. & Baasov, T. When proteins start to make sense: fine-tuning of aminoglycosides for PTC suppression therapy. *MedChemComm* **5**, 1092-1105 (2014).
161. MANUVAKHOVA, M., Keeling, K. & BEDWELL, D.M. Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system. *Rna* **6**, 1044-1055 (2000).
162. Wang, D. et al. The designer aminoglycoside NB84 significantly reduces glycosaminoglycan accumulation associated with MPS IH in the Idua-W392X mouse. *Molecular genetics and metabolism* **105**, 116-125 (2012).
163. Matalonga, L. et al. Effect of readthrough treatment in fibroblasts of patients affected by lysosomal diseases caused by premature termination codons. *Neurotherapeutics* **12**, 874-886 (2015).
164. Gómez-Grau, M. et al. Evaluation of aminoglycoside and non-aminoglycoside compounds for stop-codon readthrough therapy in four lysosomal storage diseases. *PLoS One* **10** (2015).
165. Buck, N.E., Wood, L.R., Hamilton, N.J., Bennett, M.J. & Peters, H.L. Treatment of a methylmalonyl-CoA mutase stopcodon mutation. *Biochemical and biophysical research communications* **427**, 753-757 (2012).
166. Lombardi, S. et al. Translational readthrough of GLA nonsense mutations suggests dominant-negative effects exerted by the interaction of wild-type and missense variants. *RNA biology* **17**, 254-263 (2020).
167. Fazzari, M., Frasca, A., Bifari, F. & Landsberger, N. Aminoglycoside drugs induce efficient read-through of CDKL5 nonsense mutations, slightly restoring its kinase activity. *RNA biology* **16**, 1414-1423 (2019).
168. Ferrarese, M. et al. Secretion of wild-type factor IX upon readthrough over F9 pre-peptide nonsense mutations causing hemophilia B. *Human mutation* **39**, 702-708 (2018).
169. Bartolomeo, R., Polishchuk, E.V., Volpi, N., Polishchuk, R.S. & Auricchio, A. Pharmacological read-through of nonsense ARSB mutations as a potential therapeutic approach for mucopolysaccharidosis VI. *Journal of inherited metabolic disease* **36**, 363-371 (2013).
170. Miller, J.N., Kovacs, A.D. & Pearce, D.A. The novel Cln1R151X mouse model of infantile neuronal ceroid lipofuscinosis (INCL) for testing nonsense suppression therapy. *Human molecular genetics* **24**, 185-196 (2015).
171. Ho, G., Reichardt, J. & Christodoulou, J. In vitro read-through of phenylalanine hydroxylase (PAH) nonsense mutations using aminoglycosides: a potential therapy for phenylketonuria. *Journal of inherited metabolic disease* **36**, 955-959 (2013).
172. Wilschanski, M. et al. Chronic ataluren (PTC124) treatment of nonsense mutation cystic fibrosis. *European Respiratory Journal* **38**, 59-69 (2011).
173. Konstan, M., Kerem, E. & DeBoeck, K. Ataluren treatment of patients with nonsense mutation cystic fibrosis. *Lancet Respir Med. Early Online Publication*, 70100-6 (2014).
174. Konstan, M. et al. Efficacy and safety of ataluren in patients with nonsense-mutation cystic fibrosis not receiving chronic inhaled aminoglycosides: the international, randomized, double-blind, placebo-controlled Ataluren Confirmatory Trial in Cystic Fibrosis (ACT CF). *Journal of Cystic Fibrosis* (2020).
175. EitanKerem, M.W.K. et al. A randomized placebo-controlled trial of ataluren for the treatment of nonsense mutation cystic fibrosis. *The Lancet. Respiratory medicine* **2**, 539 (2014).

176. Blanchet, S., Cornu, D., Argentini, M. & Namy, O. New insights into the incorporation of natural suppressor tRNAs at stop codons in Saccharomyces cerevisiae. *Nucleic acids research* **42**, 10061-10072 (2014).
177. Pranke, I. et al. Factors influencing readthrough therapy for frequent cystic fibrosis premature termination codons. *ERJ open research* **4**, 00080-2017 (2018).
178. Tutone, M. et al. Pharmacophore-Based Design of New Chemical Scaffolds as Translational Readthrough-Inducing Drugs (TRIDs). *ACS Medicinal Chemistry Letters* **11**, 747-753 (2020).
179. Yue, W.W., Mackinnon, S. & Bezerra, G.A. Substrate reduction therapy for inborn errors of metabolism. *Emerging Topics in Life Sciences* **3**, 63-73 (2019).
180. Coutinho, M.F., Santos, J.I. & Alves, S. Less is more: substrate reduction therapy for lysosomal storage disorders. *International journal of molecular sciences* **17**, 1065 (2016).
181. Cox, T. et al. Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT 918) to decrease substrate biosynthesis. *Lancet* **355**, 1481-5 (2000).
182. Platt, F.M., Neises, G.R., Karlsson, G.B., Dwek, R.A. & Butters, T.D. N-butyldeoxygalactonojirimycin inhibits glycolipid biosynthesis but does not affect N-linked oligosaccharide processing. *J Biol Chem* **269**, 27108-14 (1994).
183. Mistry, P.K. et al. Glucocerebrosidase 2 gene deletion rescues type 1 Gaucher disease. *Proc Natl Acad Sci U S A* **111**, 4934-9 (2014).
184. Fecarotta, S. et al. Long term follow-up to evaluate the efficacy of miglustat treatment in Italian patients with Niemann-Pick disease type C. *Orphanet J Rare Dis* **10**, 22 (2015).
185. Nietupski, J.B. et al. Iminosugar-based inhibitors of glucosylceramide synthase prolong survival but paradoxically increase brain glucosylceramide levels in Niemann-Pick C mice. *Mol Genet Metab* **105**, 621-8 (2012).
186. Cox, T.M. et al. Eliglustat compared with imiglucerase in patients with Gaucher's disease type 1 stabilised on enzyme replacement therapy: a phase 3, randomised, open-label, non-inferiority trial. *Lancet* **385**, 2355-62 (2015).
187. Mistry, P.K. et al. Outcomes after 18 months of eliglustat therapy in treatment-naive adults with Gaucher disease type 1: The phase 3 ENGAGE trial. *Am J Hematol* **92**, 1170-1176 (2017).
188. Balwani, M. et al. Recommendations for the use of eliglustat in the treatment of adults with Gaucher disease type 1 in the United States. *Mol Genet Metab* **117**, 95-103 (2016).
189. Guerard, N. et al. Lucerastat, an Iminosugar for Substrate Reduction Therapy: Tolerability, Pharmacodynamics, and Pharmacokinetics in Patients With Fabry Disease on Enzyme Replacement. *Clin Pharmacol Ther* **103**, 703-711 (2018).
190. Lindstedt, S., Holme, E., Lock, E.A., Hjalmarson, O. & Strandvik, B. Treatment of hereditary tyrosinaemia type I by inhibition of 4-hydroxyphenylpyruvate dioxygenase. *Lancet* **340**, 813-7 (1992).
191. Malinova, V., Wegrzyn, G. & Narajczyk, M. The use of elevated doses of genistein-rich soy extract in the gene expression-targeted isoflavone therapy for Sanfilippo disease patients. *JIMD Rep* **5**, 21-5 (2012).
192. Ranganath, L.R., Jarvis, J.C. & Gallagher, J.A. Recent advances in management of alkaptonuria (invited review; best practice article). *Journal of Clinical Pathology* **66**, 367-373 (2013).
193. Brunetti-Pierri, N. et al. Phenylbutyrate therapy for maple syrup urine disease. *Hum Mol Genet* **20**, 631-40 (2011).
194. Köse, M., Canda, E., Kagnici, M., Uçar, S.K. & Çoker, M. A Patient with MSUD: Acute Management with Sodium Phenylacetate/Sodium Benzoate and Sodium Phenylbutyrate. *Case Rep Pediatr* **2017**, 1045031 (2017).
195. AcerTherapeutics. ACER-001 for Maple Syrup Urine Disease (MSUD) (https://www.acertx.com/rare-disease-research/acer-001-for-maple-syrup-urine-disease-msud/).
196. García, M.I., de la Parra, A., Arias, C., Arredondo, M. & Cabello, J.F. Long-term cognitive functioning in individuals with tyrosinemia type 1 treated with nitisinone and protein-restricted diet. *Molecular genetics and metabolism reports* **11**, 12-16 (2017).
197. Khedr, M. et al. Nitisinone causes acquired tyrosinosis in alkaptonuria. *Journal of Inherited Metabolic Disease* (2020).
198. White, A. & M, C.T. Nitisinone-Induced Keratopathy in Alkaptonuria: A Challenging Diagnosis Despite Clinical Suspicion. *JIMD Rep* **40**, 7-9 (2018).
199. Matoori, S. & Leroux, J.C. Recent advances in the treatment of hyperammonemia. *Adv Drug Deliv Rev* **90**, 55-68 (2015).
200. Laemmle, A., Stricker, T. & Haberle, J. Switch from Sodium Phenylbutyrate to Glycerol Phenylbutyrate Improved Metabolic Stability in an Adolescent with Ornithine Transcarbamylase Deficiency. *JIMD Rep* **31**, 11-14 (2017).
201. Valayannopoulos, V. et al. Carglumic acid enhances rapid ammonia detoxification in classical organic acidurias with a favourable risk-benefit profile: a retrospective observational study. *Orphanet J Rare Dis* **11**, 32 (2016).

202. Ribas, G.S., Vargas, C.R. & Wajner, M. L-carnitine supplementation as a potential antioxidant therapy for inherited neurometabolic disorders. *Gene* **533**, 469-76 (2014).
203. Naglak, M., Salvo, R., Madsen, K., Dembure, P. & Elsas, L. The treatment of isovaleric acidemia with glycine supplement. *Pediatr Res* **24**, 9-13 (1988).
204. Koopman, W.J. et al. Mitochondrial disorders in children: toward development of small-molecule treatment strategies. *EMBO molecular medicine* **8**, 311-327 (2016).
205. Nightingale, H., Pfeffer, G., Bargiela, D., Horvath, R. & Chinnery, P.F. Emerging therapies for mitochondrial disorders. *Brain* **139**, 1633-1648 (2016).
206. Burlina, A. et al. The potential role of gut microbiota and its modulators in the management of propionic and methylmalonic acidemia. *Expert Opinion on Orphan Drugs* **6**, 683-692 (2018).
207. Colonetti, K., Roesch, L.F. & Schwartz, I.V.D. The microbiome and inborn errors of metabolism: Why we should look carefully at their interplay? *Genetics and molecular biology* **41**, 515-532 (2018).
208. Tatti, M. et al. BCM-95 and (2-hydroxypropyl)-β-cyclodextrin reverse autophagy dysfunction and deplete stored lipids in Sap C-deficient fibroblasts. *Human molecular genetics* **24**, 4198-4211 (2015).
209. Williams, I.M. et al. Improved neuroprotection using miglustat, curcumin and ibuprofen as a triple combination therapy in Niemann–Pick disease type C1 mice. *Neurobiology of disease* **67**, 9-17 (2014).
210. Cantz, T., Sharma, A.D. & Ott, M. Concise Review: Cell Therapies for Hereditary Metabolic Liver Diseases—Concepts, Clinical Results, and Future Developments. *Stem Cells* **33**, 1055-1062 (2015).
211. Najimi, M., Defresne, F. & Sokal, E.M. Concise review: updated advances and current challenges in cell therapy for inborn liver metabolic defects. *Stem cells translational medicine* **5**, 1117-1125 (2016).
212. Fox, I.J. et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* **338**, 1422-6 (1998).
213. Ambrosino, G. et al. Isolated hepatocyte transplantation for Crigler-Najjar syndrome type 1. *Cell transplantation* **14**, 151-157 (2005).
214. Muraca, M. et al. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. *The Lancet* **359**, 317-318 (2002).
215. Puppi, J. et al. Improving the techniques for human hepatocyte transplantation: report from a consensus meeting in London. *Cell transplantation* **21**, 1-10 (2012).
216. Komori, J., Boone, L., DeWard, A., Hoppo, T. & Lagasse, E. The mouse lymph node as an ectopic transplantation site for multiple tissues. *Nature biotechnology* **30**, 976 (2012).
217. Takebe, T. et al. Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* **499**, 481-484 (2013).
218. Soto-Gutierrez, A. et al. A whole-organ regenerative medicine approach for liver replacement. *Tissue Engineering Part C: Methods* **17**, 677-686 (2011).
219. Si-Tayeb, K. et al. Highly efficient generation of human hepatocyte–like cells from induced pluripotent stem cells. *Hepatology* **51**, 297-305 (2010).
220. Touboul, T. et al. Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. *Hepatology* **51**, 1754-1765 (2010).
221. Huang, P. et al. Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. *nature* **475**, 386-389 (2011).
222. Huang, P. et al. Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. *Cell stem cell* **14**, 370-384 (2014).
223. Du, Y. et al. Human hepatocytes with drug metabolic function induced from fibroblasts by lineage reprogramming. *Cell stem cell* **14**, 394-403 (2014).
224. Ordonez, M.P. & Goldstein, L.S. in Seminars in liver disease 298-306 (Thieme Medical Publishers, 2012).
225. Paganelli, M. in Pediatric Hepatology and Liver Transplantation 781-797 (Springer, 2019).
226. Dobbelaere, D., Semts, F. & McKiernam, P. Phase I/II multicenter trial of liver derived mesenchymal stem cells (HepastemR) for the treatment of urea cycle disorder and Crigler Najjar syndrome interim analysis at 6 months post infusion. *J Inherited Metab Dis* **37**, O-027 (2014).
227. Sharp, P.A. The centrality of RNA. *Cell* **136**, 577-580 (2009).
228. Cooper, T.A., Wan, L. & Dreyfuss, G. RNA and disease. *Cell* **136**, 777-793 (2009).
229. O'Rourke, J.R. & Swanson, M.S. Mechanisms of RNA-mediated disease. *Journal of Biological Chemistry* **284**, 7419-7423 (2009).
230. Wang, G. et al. Variation in the miRNA-433 binding site of FGF20 confers risk for Parkinson disease by overexpression of α-synuclein. *The American Journal of Human Genetics* **82**, 283-289 (2008).

231. Calin, G.A. et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proceedings of the national academy of sciences* **99**, 15524-15529 (2002).
232. Hannon, G.J. RNA interference. *nature* **418**, 244-251 (2002).
233. Nair, J.K. et al. Multivalent N-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. *Journal of the American Chemical Society* **136**, 16958-16961 (2014).
234. Crooke, S.T., Witztum, J.L., Bennett, C.F. & Baker, B.F. RNA-targeted therapeutics. *Cell metabolism* **27**, 714-739 (2018).
235. Bonetta, L. RNA-based therapeutics: ready for delivery? *Cell* **136**, 581-584 (2009).
236. Bumcrot, D., Manoharan, M., Koteliansky, V. & Sah, D.W. RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nature chemical biology* **2**, 711-719 (2006).
237. Crooke, S.T. Antisense drug technology: principles, strategies, and applications (CRC press, 2007).
238. Juliano, R., Alam, M.R., Dixit, V. & Kang, H. Mechanisms and strategies for effective delivery of antisense and siRNA oligonucleotides. *Nucleic acids research* **36**, 4158-4171 (2008).
239. Castanotto, D. & Rossi, J.J. The promises and pitfalls of RNA-interference-based therapeutics. *Nature* **457**, 426-433 (2009).
240. Bennett, C.F. & Swayze, E.E. RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annual review of pharmacology and toxicology* **50**, 259-293 (2010).
241. Kastelein, J.J. et al. CLINICAL PERSPECTIVE. *Circulation* **114**, 1729-1735 (2006).
242. Pardi, N. et al. Zika virus protection by a single low-dose nucleoside-modified mRNA vaccination. *Nature* **543**, 248-251 (2017).
243. Pardi, N. et al. Nucleoside-modified mRNA immunization elicits influenza virus hemagglutinin stalk-specific antibodies. *Nature communications* **9**, 1-12 (2018).
244. Jackson, L.A. et al. An mRNA vaccine against SARS-CoV-2—preliminary report. *New England Journal of Medicine* **383**, 1920-1931 (2020).
245. Mulligan, M.J. et al. Phase I/II study of COVID-19 RNA vaccine BNT162b1 in adults. *Nature*, 1-5 (2020).
246. McNamara, M.A., Nair, S.K. & Holl, E.K. RNA-based vaccines in cancer immunotherapy. *Journal of immunology research* **2015** (2015).
247. An, D. et al. Systemic messenger RNA therapy as a treatment for methylmalonic acidemia. *Cell reports* **21**, 3548-3558 (2017).
248. Kukreja, A. et al. Systemic alanine glyoxylate aminotransferase mRNA improves glyoxylate metabolism in a mouse model of primary hyperoxaluria type 1. *Nucleic acid therapeutics* **29**, 104-113 (2019).
249. Roseman, D.S. et al. G6PC mRNA therapy positively regulates fasting blood glucose and decreases liver abnormalities in a mouse model of glycogen storage disease 1a. *Molecular Therapy* **26**, 814-821 (2018).
250. Cao, J. et al. mRNA therapy improves metabolic and behavioral abnormalities in a murine model of citrin deficiency. *Molecular Therapy* **27**, 1242-1251 (2019).
251. Jiang, L. et al. Systemic messenger RNA as an etiological treatment for acute intermittent porphyria. *Nature medicine* **24**, 1899-1909 (2018).
252. Greig, J.A. et al. in MOLECULAR THERAPY 16-16 (CELL PRESS 50 HAMPSHIRE ST, FLOOR 5, CAMBRIDGE, MA 02139 USA, 2019).
253. Truong, B. et al. Lipid nanoparticle-targeted mRNA therapy as a treatment for the inherited metabolic liver disorder arginase deficiency. *Proceedings of the National Academy of Sciences* **116**, 21150-21159 (2019).
254. Wang, L. et al. in MOLECULAR THERAPY 182-182 (CELL PRESS 50 HAMPSHIRE ST, FLOOR 5, CAMBRIDGE, MA 02139 USA, 2019).
255. Cao, J. et al. in MOLECULAR THERAPY 109-110 (CELL PRESS 50 HAMPSHIRE ST, FLOOR 5, CAMBRIDGE, MA 02139 USA, 2020).
256. An, D. et al. Long-term efficacy and safety of mRNA therapy in two murine models of methylmalonic acidemia. *EBioMedicine* **45**, 519-528 (2019).
257. Rosenberg, S.A. et al. Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med* **323**, 570-8 (1990).
258. Raper, S.E. et al. A pilot study of in vivo liver-directed gene transfer with an adenoviral vector in partial ornithine transcarbamylase deficiency. *Hum Gene Ther* **13**, 163-75 (2002).
259. Hacein-Bey-Abina, S. et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**, 415-9 (2003).
260. Howe, S.J. et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest* **118**, 3143-50 (2008).

261. Hacein-Bey-Abina, S. et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest* **118**, 3132-42 (2008).
262. Hacein-Bey-Abina, S. et al. Efficacy of gene therapy for X-linked severe combined immunodeficiency. *New England Journal of Medicine* **363**, 355-364 (2010).
263. Ott, M.G. et al. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nature medicine* **12**, 401-409 (2006).
264. Stein, S. et al. Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nature medicine* **16**, 198-204 (2010).
265. Dunbar, C.E. et al. Gene therapy comes of age. *Science* **359**, eaan4672 (2018).
266. Bainbridge, J.W. et al. Effect of gene therapy on visual function in Leber's congenital amaurosis. *New England Journal of Medicine* **358**, 2231-2239 (2008).
267. Cideciyan, A.V. et al. Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics. *Proceedings of the National Academy of Sciences* **105**, 15112-15117 (2008).
268. Maguire, A.M. et al. Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med* **358**, 2240-8 (2008).
269. Nathwani, A.C. et al. Long-term safety and efficacy following systemic administration of a self-complementary AAV vector encoding human FIX pseudotyped with serotype 5 and 8 capsid proteins. *Mol Ther* **19**, 876-85 (2011).
270. Manno, C.S. et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* **12**, 342-7 (2006).
271. Nathwani, A.C. et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* **371**, 1994-2004 (2014).
272. George, L.A. et al. Hemophilia B gene therapy with a high-specific-activity factor IX variant. *New England Journal of Medicine* **377**, 2215-2227 (2017).
273. Cavazzana-Calvo, M. et al. Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia. *Nature* **467**, 318-322 (2010).
274. Aiuti, A. et al. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science* **341**, 1233151 (2013).
275. Cartier, N. et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* **326**, 818-23 (2009).
276. Biffi, A. et al. Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science* **341**, 1233158 (2013).
277. Couzin-Frankel, J. Breakthrough of the year 2013. *Cancer Immunother Sci* **342**, 1432-3 (2013).
278. Gottlieb, D.S. (ed. Terry, M.) (BioSpace, 2018).
279. Wang, D. & Gao, G. State-of-the-art human gene therapy: part Ii. gene therapy strategies and applications. *Discovery medicine* **18**, 151 (2014).
280. Ginocchio, V.M., Ferla, R., Auricchio, A. & Brunetti-Pierri, N. Current status on clinical development of adeno-associated virus-mediated liver-directed gene therapy for inborn errors of metabolism. *Human gene therapy* **30**, 1204-1210 (2019).
281. Brunetti-Pierri, N. in Handbook of Clinical Adult Genetics and Genomics 463-477 (Elsevier, 2020).
282. Poletti, V. & Biffi, A. Gene-based approaches to inherited neurometabolic diseases. *Human Gene Therapy* **30**, 1222-1235 (2019).
283. Muro, A.F., D'Antiga, L. & Mingozzi, F. in Pediatric Hepatology and Liver Transplantation 799-829 (Springer, 2019).
284. Zhou, Z. et al. Nonviral cancer gene therapy: Delivery cascade and vector nanoproperty integration. *Advanced drug delivery reviews* **115**, 115-154 (2017).
285. Lin, G. et al. Non-viral gene therapy using multifunctional nanoparticles: Status, challenges, and opportunities. *Coordination Chemistry Reviews* **374**, 133-152 (2018).
286. Jayant, R.D. et al. Current status of non-viral gene therapy for CNS disorders. *Expert opinion on drug delivery* **13**, 1433-1445 (2016).
287. Hardee, C.L., Arévalo-Soliz, L.M., Hornstein, B.D. & Zechiedrich, L. Advances in non-viral DNA vectors for gene therapy. *Genes* **8**, 65 (2017).
288. Picanço-Castro, V. et al. Emerging patent landscape for non-viral vectors used for gene therapy. *Nature Biotechnology* **38**, 151-157 (2020).
289. Thompson, A.A. et al. Gene Therapy in Patients with Transfusion-Dependent β-Thalassemia. *N Engl J Med* **378**, 1479-1493 (2018).
290. Ribeil, J.-A. et al. Gene therapy in a patient with sickle cell disease. *New England Journal of Medicine* **376**, 848-855 (2017).
291. Eichler, F. et al. Hematopoietic stem-cell gene therapy for cerebral adrenoleukodystrophy. *New England Journal of Medicine* **377**, 1630-1638 (2017).

292. Zheng, Y. et al. Treatment of the mouse model of mucopolysaccharidosis I with retrovirally transduced bone marrow. *Molecular genetics and metabolism* **79**, 233-244 (2003).
293. Wang, D. et al. Reprogramming erythroid cells for lysosomal enzyme production leads to visceral and CNS cross-correction in mice with Hurler syndrome. *Proceedings of the National Academy of Sciences* **106**, 19958-19963 (2009).
294. Visigalli, I. et al. Gene therapy augments the efficacy of hematopoietic cell transplantation and fully corrects mucopolysaccharidosis type I phenotype in the mouse model. *Blood, The Journal of the American Society of Hematology* **116**, 5130-5139 (2010).
295. Sergijenko, A. et al. Myeloid/Microglial driven autologous hematopoietic stem cell gene therapy corrects a neuronopathic lysosomal disease. *Molecular Therapy* **21**, 1938-1949 (2013).
296. Ellison, S.M. et al. Pre-clinical safety and efficacy of lentiviral vector-mediated ex vivo stem cell gene therapy for the treatment of mucopolysaccharidosis IIIA. *Molecular Therapy-Methods & Clinical Development* **13**, 399-413 (2019).
297. Squeri, G. et al. Targeting a pre-existing anti-transgene T cell response for effective gene therapy of MPS-I in the mouse model of the disease. *Molecular Therapy* **27**, 1215-1227 (2019).
298. Rocca, C.J. & Cherqui, S. Potential use of stem cells as a therapy for cystinosis. *Pediatric Nephrology* **34**, 965-973 (2019).
299. Grossman, M. et al. Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. *Nature genetics* **6**, 335-341 (1994).
300. Nguyen, T.H. et al. Ex vivo lentivirus transduction and immediate transplantation of uncultured hepatocytes for treating hyperbilirubinemic Gunn rat. *Transplantation* **82**, 794-803 (2006).
301. Nguyen, T.H. et al. Ex vivo liver-directed gene therapy for the treatment of metabolic diseases: advances in hepatocyte transplantation and retroviral vectors. *Curr Gene Ther* **9**, 136-49 (2009).
302. Famulari, E.S. et al. Human liver stem cells express UGT1A1 and improve phenotype of immunocompromised Crigler Najjar syndrome type I mice. *Scientific Reports* **10**, 887 (2020).
303. David, R.M. & Doherty, A.T. Viral vectors: the road to reducing genotoxicity. *Toxicological Sciences* **155**, 315-325 (2017).
304. Calcedo, R., Vandenberghe, L.H., Gao, G., Lin, J. & Wilson, J.M. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J Infect Dis* **199**, 381-90 (2009).
305. Verdera, H.C., Kuranda, K. & Mingozzi, F. AAV vector immunogenicity in humans: A long journey to successful gene transfer. *Molecular Therapy* **28**, 723-746 (2020).
306. Boutin, S. et al. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum Gene Ther* **21**, 704-12 (2010).
307. Erles, K., Sebokova, P. & Schlehofer, J.R. Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). *J Med Virol* **59**, 406-11 (1999).
308. Veron, P. et al. Humoral and cellular capsid-specific immune responses to adeno-associated virus type 1 in randomized healthy donors. *The Journal of Immunology* **188**, 6418-6424 (2012).
309. Leborgne, C. et al. Prevalence and long-term monitoring of humoral immunity against adeno-associated virus in Duchenne Muscular Dystrophy patients. *Cellular immunology* **342**, 103780 (2019).
310. Kuranda, K. et al. Exposure to wild-type AAV drives distinct capsid immunity profiles in humans. *J Clin Invest* **128**, 5267-5279 (2018).
311. Ronzitti, G., Gross, D.-A. & Mingozzi, F. Human Immune Responses to Adeno-Associated Virus (AAV) Vectors. *Frontiers in Immunology* **11** (2020).
312. Scallan, C.D. et al. Human immunoglobulin inhibits liver transduction by AAV vectors at low AAV2 neutralizing titers in SCID mice. *Blood* **107**, 1810-1817 (2006).
313. Jiang, H. et al. Effects of transient immunosuppression on adenoassociated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy. *Blood* **108**, 3321-3328 (2006).
314. Manno, C.S. et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* **101**, 2963-72 (2003).
315. Fitzpatrick, Z. et al. Influence of Pre-existing Anti-capsid Neutralizing and Binding Antibodies on AAV Vector Transduction. *Mol Ther Methods Clin Dev* **9**, 119-129 (2018).
316. Wellman, J.A. et al. in MOLECULAR THERAPY S28-S29 (NATURE PUBLISHING GROUP 75 VARICK ST, 9TH FLR, NEW YORK, NY 10013-1917 USA, 2012).
317. Mingozzi, F. et al. Overcoming preexisting humoral immunity to AAV using capsid decoys. *Sci Transl Med* **5**, 194ra92 (2013).
318. Mingozzi, F. et al. Modulation of tolerance to the transgene product in a nonhuman primate model of AAV-mediated gene transfer to liver. *Blood* **110**, 2334-2341 (2007).
319. Mingozzi, F. et al. Pharmacological modulation of humoral immunity in a nonhuman primate model of AAV gene transfer for hemophilia B. *Molecular Therapy* **20**, 1410-1416 (2012).

320. Mingozzi, F. et al. CD8+ T-cell responses to adeno-associated virus capsid in humans. *Nature Medicine* **13**, 419-422 (2007).
321. Li, H. et al. Capsid-specific T-cell Responses to Natural Infections With Adeno-associated Viruses in Humans Differ From Those of Nonhuman Primates. *Molecular Therapy* **19**, 2021-2030 (2011).
322. Xiang, Z. et al. The effect of CpG sequences on capsid-specific CD8+ T cell responses to AAV vector gene transfer. *Molecular Therapy* **28**, 771-783 (2020).
323. Chao, H., Monahan, P.E., Liu, Y., Samulski, R.J. & Walsh, C.E. Sustained and complete phenotype correction of hemophilia B mice following intramuscular injection of AAV1 serotype vectors. *Molecular Therapy* **4**, 217-222 (2001).
324. Monahan, P. et al. Direct intramuscular injection with recombinant AAV vectors results in sustained expression in a dog model of hemophilia. *Gene therapy* **5**, 40-49 (1998).
325. Flotte, T.R. et al. Phase 2 clinical trial of a recombinant adeno-associated viral vector expressing α1-antitrypsin: interim results. *Human gene therapy* **22**, 1239-1247 (2011).
326. Mueller, C. et al. Human Treg responses allow sustained recombinant adeno-associated virus–mediated transgene expression. *The Journal of clinical investigation* **123**, 5310-5318 (2013).
327. Stroes, E.S. et al. Intramuscular administration of AAV1-lipoprotein lipaseS447X lowers triglycerides in lipoprotein lipase–deficient patients. *Arteriosclerosis, thrombosis, and vascular biology* **28**, 2303-2304 (2008).
328. Gaudet, D. et al. Efficacy and long-term safety of alipogene tiparvovec (AAV1-LPLS447X) gene therapy for lipoprotein lipase deficiency: an open-label trial. *Gene Ther* **20**, 361-9 (2013).
329. Scott, L.J. Alipogene tiparvovec: a review of its use in adults with familial lipoprotein lipase deficiency. *Drugs* **75**, 175-182 (2015).
330. Ylä-Herttuala, S. Endgame: glybera finally recommended for approval as the first gene therapy drug in the European union. *Molecular Therapy* **20**, 1831-1832 (2012).
331. Bowles, D.E. et al. Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. *Molecular Therapy* **20**, 443-455 (2012).
332. Buchlis, G. et al. Factor IX expression in skeletal muscle of a severe hemophilia B patient 10 years after AAV-mediated gene transfer. *Blood, The Journal of the American Society of Hematology* **119**, 3038-3041 (2012).
333. Le Guiner, C. et al. Long-term microdystrophin gene therapy is effective in a canine model of Duchenne muscular dystrophy. *Nature communications* **8**, 1-15 (2017).
334. Mendell, J.R. et al. A phase 1/2a follistatin gene therapy trial for becker muscular dystrophy. *Molecular Therapy* **23**, 192-201 (2015).
335. Mingozzi, F. et al. AAV-1–mediated gene transfer to skeletal muscle in humans results in dose-dependent activation of capsid-specific T cells. *Blood* **114**, 2077-2086 (2009).
336. VandenDriessche, T. Muscling through AAV immunity. *Blood, The Journal of the American Society of Hematology* **114**, 2009-2010 (2009).
337. Hinderer, C. et al. Severe toxicity in nonhuman primates and piglets following high-dose intravenous administration of an adeno-associated virus vector expressing human SMN. *Human gene therapy* **29**, 285-298 (2018).
338. Kaiser, J. (American Association for the Advancement of Science, 2018).
339. Audentes. (https://www.joshuafrase.org/get-involved/recensus-study.php, 2020).
340. Wilson, J.M. & Flotte, T.R. Moving Forward after Two Deaths in a Gene Therapy Trial of Myotubular Myopathy: To learn from these tragedies, the scientific community must commit to full transparency and cooperation. *Genetic Engineering & Biotechnology News* **40**, 14, 16 (2020).
341. Mendell, J.R. et al. Single-dose gene-replacement therapy for spinal muscular atrophy. *New England Journal of Medicine* **377**, 1713-1722 (2017).
342. Petit, L., Khanna, H. & Punzo, C. Advances in gene therapy for diseases of the eye. *Human gene therapy* **27**, 563-579 (2016).
343. Bennett, J. Taking stock of retinal gene therapy: looking back and moving forward. *Molecular Therapy* **25**, 1076-1094 (2017).
344. Auricchio, A., Smith, A.J. & Ali, R.R. The future looks brighter after 25 years of retinal gene therapy. *Human gene therapy* **28**, 982-987 (2017).
345. Russell, S. et al. Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. *The Lancet* **390**, 849-860 (2017).
346. Hocquemiller, M., Giersch, L., Audrain, M., Parker, S. & Cartier, N. Adeno-associated virus-based gene therapy for CNS diseases. *Human gene therapy* **27**, 478-496 (2016).
347. Foust, K.D. et al. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nature biotechnology* **27**, 59-65 (2009).
348. Duque, S. et al. Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. *Molecular Therapy* **17**, 1187-1196 (2009).

349. Zhang, H. et al. Several rAAV vectors efficiently cross the blood–brain barrier and transduce neurons and astrocytes in the neonatal mouse central nervous system. *Molecular Therapy* **19**, 1440-1448 (2011).
350. Yang, B. et al. 526. Intravasuclar Delivery of RAAVRH. 8 Generates Widespreading Transduction of Neuronal and Glial Cell Types in the Adult Mouse Central Nervous System. *Molecular Therapy* **20**, 1 (2012).
351. Foust, K.D. et al. Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. *Nature biotechnology* **28**, 271-274 (2010).
352. Stoica, L. et al. Adeno-associated virus–delivered artificial microRNA extends survival and delays paralysis in an amyotrophic lateral sclerosis mouse model. *Annals of neurology* **79**, 687-700 (2016).
353. Foust, K.D. et al. Therapeutic AAV9-mediated suppression of mutant SOD1 slows disease progression and extends survival in models of inherited ALS. *Molecular Therapy* **21**, 2148-2159 (2013).
354. Borel, F. et al. Therapeutic rAAVrh10 mediated SOD1 silencing in adult SOD1 G93A mice and nonhuman primates. *Human gene therapy* **27**, 19-31 (2016).
355. Ahmed, S.S. et al. A single intravenous rAAV injection as late as P20 achieves efficacious and sustained CNS Gene therapy in Canavan mice. *Molecular Therapy* **21**, 2136-2147 (2013).
356. Sondhi, D. et al. Long-term expression and safety of administration of AAVrh. 10hCLN2 to the brain of rats and nonhuman primates for the treatment of late infantile neuronal ceroid lipofuscinosis. *Human Gene Therapy, Part B: Methods* **23**, 324-335 (2012).
357. Gessler, D.J. et al. Redirecting N-acetylaspartate metabolism in the central nervous system normalizes myelination and rescues Canavan disease. *JCI insight* **2** (2017).
358. Weismann, C.M. et al. Systemic AAV9 gene transfer in adult GM1 gangliosidosis mice reduces lysosomal storage in CNS and extends lifespan. *Human molecular genetics* **24**, 4353-4364 (2015).
359. Fu, H., DiRosario, J., Killedar, S., Zaraspe, K. & McCarty, D.M. Correction of neurological disease of mucopolysaccharidosis IIIB in adult mice by rAAV9 trans-blood–brain barrier gene delivery. *Molecular Therapy* **19**, 1025-1033 (2011).
360. Gong, Y. et al. Intrathecal adeno-associated viral vector-mediated gene delivery for adrenomyeloneuropathy. *Human gene therapy* **30**, 544-555 (2019).
361. Biferi, M.G. et al. A new AAV10-U7-mediated gene therapy prolongs survival and restores function in an ALS mouse model. *Molecular Therapy* **25**, 2038-2052 (2017).
362. Colella, P., Ronzitti, G. & Mingozzi, F. Emerging issues in AAV-mediated in vivo gene therapy. *Molecular Therapy-Methods & Clinical Development* **8**, 87-104 (2018).
363. Keeler, G.D., Markusic, D.M. & Hoffman, B.E. Liver induced transgene tolerance with AAV vectors. *Cellular immunology* **342**, 103728 (2019).
364. Hinderer, C. et al. Neonatal tolerance induction enables accurate evaluation of gene therapy for MPS I in a canine model. *Molecular genetics and metabolism* **119**, 124-130 (2016).
365. Hinderer, C. et al. Neonatal systemic AAV induces tolerance to CNS gene therapy in MPS I dogs and nonhuman primates. *Molecular Therapy* **23**, 1298-1307 (2015).
366. Rangarajan, S. et al. AAV5-Factor VIII Gene Transfer in Severe Hemophilia A. *N Engl J Med* **377**, 2519-2530 (2017).
367. Russell, D.W., Miller, A.D. & Alexander, I.E. Adeno-associated virus vectors preferentially transduce cells in S phase. *Proceedings of the National Academy of Sciences* **91**, 8915-8919 (1994).
368. McCarty, D.M., Young Jr, S.M. & Samulski, R.J. Integration of adeno-associated virus (AAV) and recombinant AAV vectors. *Annu. Rev. Genet.* **38**, 819-845 (2004).
369. Smith, R. Adeno-associated virus integration: virus versus vector. *Gene therapy* **15**, 817-822 (2008).
370. Li, H. et al. Assessing the potential for AAV vector genotoxicity in a murine model. *Blood, The Journal of the American Society of Hematology* **117**, 3311-3319 (2011).
371. Valdmanis, P.N., Lisowski, L. & Kay, M.A. rAAV-mediated tumorigenesis: still unresolved after an AAV assault. *Molecular Therapy* **20**, 2014-2017 (2012).
372. Donsante, A. et al. AAV vector integration sites in mouse hepatocellular carcinoma. *Science* **317**, 477-477 (2007).
373. Reiss, J. & Hahnewald, R. Molybdenum cofactor deficiency: mutations in GPHN, MOCS1, and MOCS2. *Human mutation* **32**, 10-18 (2011).
374. Chandler, R.J. et al. Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy. *The Journal of clinical investigation* **125**, 870-880 (2015).
375. Niemeyer, G.P. et al. Long-term correction of inhibitor-prone hemophilia B dogs treated with liver-directed AAV2-mediated factor IX gene therapy. *Blood, The Journal of the American Society of Hematology* **113**, 797-806 (2009).
376. Nguyen, G.N. et al. A long-term study of AAV gene therapy in dogs with hemophilia A identifies clonal expansions of transduced liver cells. *Nat Biotechnol* **39**, 47-55 (2021).
377. Nault, J.-C. et al. Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. *Nature genetics* **47**, 1187-1193 (2015).

378. La Bella, T. et al. Adeno-associated virus in the liver: natural history and consequences in tumour development. *Gut* **69**, 737-747 (2020).
379. Logan, G.J. et al. Identification of liver-specific enhancer–promoter activity in the 3′ untranslated region of the wild-type AAV2 genome. *Nature genetics* **49**, 1267-1273 (2017).
380. Jessup, M. et al. Calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID) a phase 2 trial of intracoronary gene therapy of sarcoplasmic reticulum Ca2+-ATPase in patients with advanced heart failure. *Circulation* **124**, 304-313 (2011).
381. Pépin, D. et al. AAV9 delivering a modified human Mullerian inhibiting substance as a gene therapy in patient-derived xenografts of ovarian cancer. *Proceedings of the National Academy of Sciences* **112**, E4418-E4427 (2015).
382. Johnson, P.R. et al. Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys. *Nature medicine* **15**, 901-906 (2009).
383. Balazs, A.B. & West, A.P. Antibody gene transfer for HIV immunoprophylaxis. *Nature immunology* **14**, 1-5 (2013).
384. Gardner, M.R. et al. AAV-expressed eCD4-Ig provides durable protection from multiple SHIV challenges. *Nature* **519**, 87-91 (2015).
385. Limberis, M.P. et al. Intranasal antibody gene transfer in mice and ferrets elicits broad protection against pandemic influenza. *Science translational medicine* **5**, 187ra72-187ra72 (2013).
386. Fuchs, S.P. et al. AAV-delivered antibody mediates significant protective effects against SIVmac239 challenge in the absence of neutralizing activity. *PLoS Pathog* **11**, e1005090 (2015).
387. Du, L. et al. A conformation-dependent neutralizing monoclonal antibody specifically targeting receptor-binding domain in Middle East respiratory syndrome coronavirus spike protein. *Journal of virology* **88**, 7045-7053 (2014).
388. Martinez-Navio, J.M. et al. Adeno-associated virus delivery of anti-HIV monoclonal antibodies can drive long-term virologic suppression. *Immunity* **50**, 567-575. e5 (2019).
389. Barbon, E. et al. Single-domain antibodies targeting antithrombin reduce bleeding in hemophilic mice with or without inhibitors. *EMBO molecular medicine* **12**, e11298 (2020).
390. Priddy, F.H. et al. Adeno-associated virus vectored immunoprophylaxis to prevent HIV in healthy adults: a phase 1 randomised controlled trial. *The Lancet HIV* **6**, e230-e239 (2019).
391. Gardner, M.R. et al. Anti-drug antibody responses impair prophylaxis mediated by AAV-delivered HIV-1 broadly neutralizing antibodies. *Molecular Therapy* **27**, 650-660 (2019).
392. Borel, F., Kay, M.A. & Mueller, C. Recombinant AAV as a platform for translating the therapeutic potential of RNA interference. *Molecular Therapy* **22**, 692-701 (2014).
393. Grimm, D. et al. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**, 537-541 (2006).
394. Abudayyeh, O.O. et al. RNA targeting with CRISPR–Cas13. *Nature* **550**, 280-284 (2017).
395. Konermann, S. et al. Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors. *Cell* **173**, 665-676. e14 (2018).
396. Chew, W.L. et al. A multifunctional AAV–CRISPR–Cas9 and its host response. *Nature methods* **13**, 868-874 (2016).
397. Hynes, A.P. et al. Widespread anti-CRISPR proteins in virulent bacteriophages inhibit a range of Cas9 proteins. *Nature communications* **9**, 1-10 (2018).
398. Yang, Y. et al. A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in newborn mice. *Nature biotechnology* **34**, 334-338 (2016).
399. Ding, Q. et al. Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. *Circulation research* **115**, 488-492 (2014).
400. Wang, L. et al. Meganuclease targeting of PCSK9 in macaque liver leads to stable reduction in serum cholesterol. *Nature biotechnology* **36**, 717-725 (2018).
401. Bakondi, B. et al. In vivo CRISPR/Cas9 gene editing corrects retinal dystrophy in the S334ter-3 rat model of autosomal dominant retinitis pigmentosa. *Molecular Therapy* **24**, 556-563 (2016).
402. Yin, H. et al. Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nature biotechnology* **32**, 551-553 (2014).
403. Yin, H. et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. *Nature biotechnology* **34**, 328-333 (2016).
404. Song, C.-Q. et al. In vivo genome editing partially restores alpha1-antitrypsin in a murine model of AAT deficiency. *Human gene therapy* **29**, 853-860 (2018).
405. Shen, S. et al. Amelioration of alpha-1 antitrypsin deficiency diseases with genome editing in transgenic mice. *Human gene therapy* **29**, 861-873 (2018).
406. Zabaleta, N. et al. CRISPR/Cas9-mediated glycolate oxidase disruption is an efficacious and safe treatment for primary hyperoxaluria type I. *Nat Commun* **9**, 5454 (2018).
407. Li, H. et al. In vivo genome editing restores haemostasis in a mouse model of haemophilia. *Nature* **475**, 217-221 (2011).

408. Anguela, X.M. et al. Robust ZFN-mediated genome editing in adult hemophilic mice. *Blood* **122**, 3283-3287 (2013).
409. Sharma, R. et al. In vivo genome editing of the albumin locus as a platform for protein replacement therapy. *Blood, The Journal of the American Society of Hematology* **126**, 1777-1784 (2015).
410. Long, C. et al. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science* **351**, 400-403 (2016).
411. Nelson, C.E. et al. In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science* **351**, 403-407 (2016).
412. Tabebordbar, M. et al. In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science* **351**, 407-411 (2016).
413. Wang, D. et al. Cas9-mediated allelic exchange repairs compound heterozygous recessive mutations in mice. *Nature biotechnology* **36**, 839-842 (2018).
414. Amoasii, L. et al. Gene editing restores dystrophin expression in a canine model of Duchenne muscular dystrophy. *Science* **362**, 86-91 (2018).
415. Ran, F.A. et al. In vivo genome editing using Staphylococcus aureus Cas9. *Nature* **520**, 186-191 (2015).
416. Kleinstiver, B.P. et al. High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* **529**, 490-495 (2016).
417. Slaymaker, I.M. et al. Rationally engineered Cas9 nucleases with improved specificity. *Science* **351**, 84-88 (2016).
418. Tsai, S.Q. & Joung, J.K. Defining and improving the genome-wide specificities of CRISPR–Cas9 nucleases. *Nature Reviews Genetics* **17**, 300-312 (2016).
419. Tebas, P. et al. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *New England Journal of Medicine* **370**, 901-910 (2014).
420. Bauer, D.E. et al. An erythroid enhancer of BCL11A subject to genetic variation determines fetal hemoglobin level. *Science* **342**, 253-257 (2013).
421. Cornu, T.I., Mussolino, C. & Cathomen, T. Refining strategies to translate genome editing to the clinic. *Nature Medicine* **23**, 415-423 (2017).
422. Jiang, J. et al. Translating dosage compensation to trisomy 21. *Nature* **500**, 296-300 (2013).
423. Rees, H.A. & Liu, D.R. Base editing: precision chemistry on the genome and transcriptome of living cells. *Nature reviews genetics* **19**, 770-788 (2018).
424. Schneller, J.L., Lee, C.M., Bao, G. & Venditti, C.P. Genome editing for inborn errors of metabolism: advancing towards the clinic. *BMC medicine* **15**, 43 (2017).
425. Novartis, N., Portfolio, G.P. & Data, F. Novartis Receives First Ever FDA Approval for a CAR-T Cell Therapy, Kymriah (TM)(CTL019), for Children and Young Adults with B-cell ALL That Is Eefractory or Has Relapsed At least Twice. (2017).
426. Fala, L. Yescarta (Axicabtagene Ciloleucel) second CAR T-cell therapy approved for patients with certain types of large B-cell lymphoma. (2018).
427. Neelapu, S.S. et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *New England Journal of Medicine* **377**, 2531-2544 (2017).
428. Maude, S.L. et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *New England Journal of Medicine* **378**, 439-448 (2018).
429. Porter, D., Frey, N., Wood, P.A., Weng, Y. & Grupp, S.A. Grading of cytokine release syndrome associated with the CAR T cell therapy tisagenlecleucel. *Journal of hematology & oncology* **11**, 35 (2018).
430. Catros, V. Next generation engineered T cells for cell therapy: from lymphoma to solid tumors. *Medecine Sciences: M/S* **35**, 316-326 (2019).
431. Nathwani, A.C. et al. Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* **107**, 2653-61 (2006).
432. Nathwani, A.C. et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med* **365**, 2357-65 (2011).
433. Miesbach, W. et al. Gene therapy with adeno-associated virus vector 5-human factor IX in adults with hemophilia B. *Blood* **131**, 1022-1031 (2018).
434. Mingozzi, F. et al. Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer. *J Clin Invest* **111**, 1347-56 (2003).
435. Dobrzynski, E. et al. Induction of antigen-specific CD4+ T-cell anergy and deletion by in vivo viral gene transfer. *Blood* **104**, 969-77 (2004).
436. Cao, O. et al. Induction and role of regulatory CD4+CD25+ T cells in tolerance to the transgene product following hepatic in vivo gene transfer. *Blood* **110**, 1132-40 (2007).
437. Ronzitti, G., Collaud, F., Laforet, P. & Mingozzi, F. Progress and challenges of gene therapy for Pompe disease. *Ann Transl Med* **7**, 287 (2019).

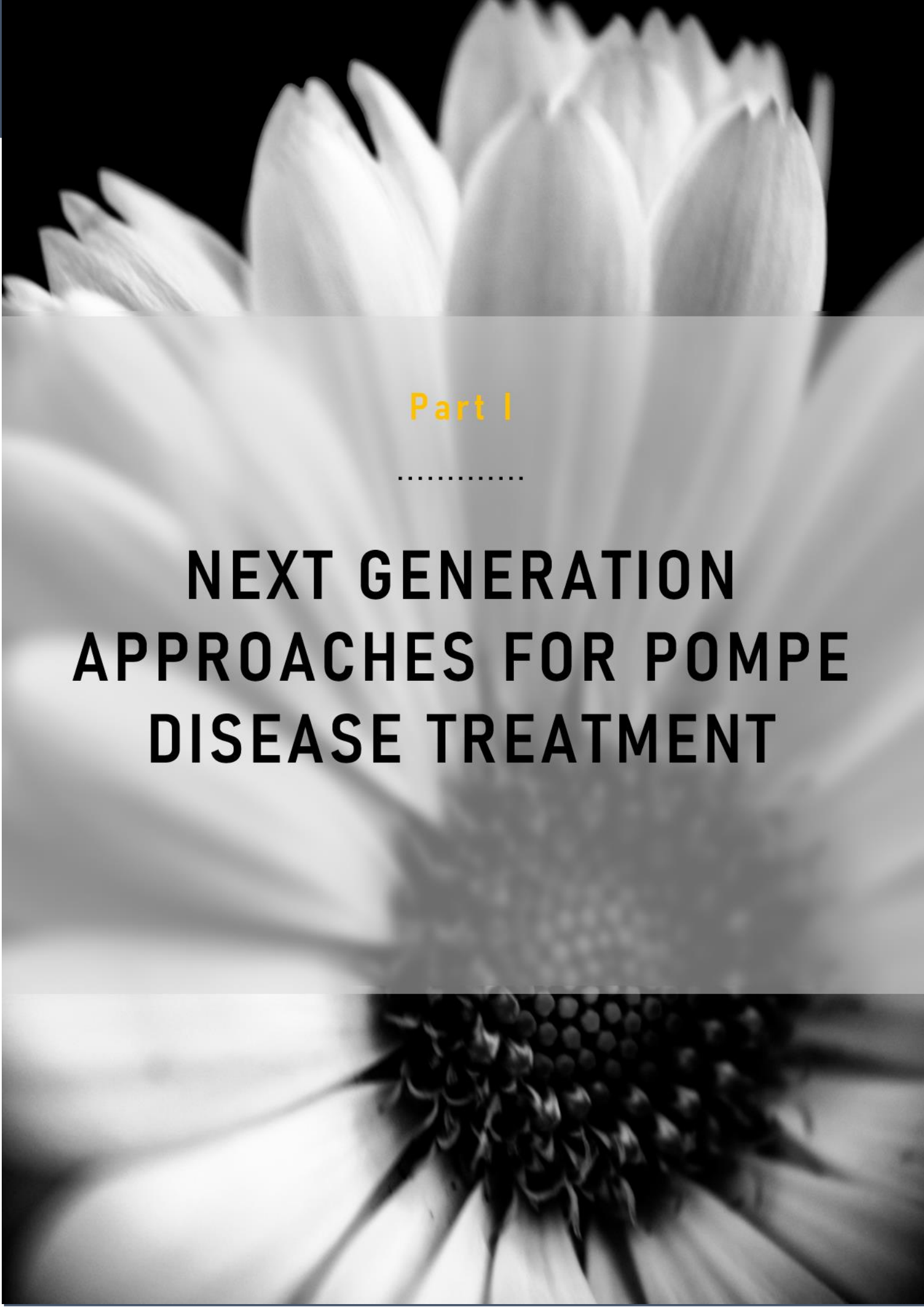
438. Nakai, H. et al. Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction in vivo. *Journal of virology* **75**, 6969-6976 (2001).
439. Wang, L., Wang, H., Bell, P., McMenamin, D. & Wilson, J.M. Hepatic gene transfer in neonatal mice by adeno-associated virus serotype 8 vector. *Hum Gene Ther* **23**, 533-9 (2012).
440. Bortolussi, G. et al. Life-long correction of hyperbilirubinemia with a neonatal liver-specific AAV-mediated gene transfer in a lethal mouse model of Crigler-Najjar Syndrome. *Hum Gene Ther* **25**, 844-55 (2014).
441. Cunningham, S.C., Dane, A.P., Spinoulas, A. & Alexander, I.E. Gene delivery to the juvenile mouse liver using AAV2/8 vectors. *Molecular Therapy* **16**, 1081-1088 (2008).
442. Junge, N., Mingozzi, F., Ott, M. & Baumann, U. Adeno-associated virus vector-based gene therapy for monogenetic metabolic diseases of the liver. *J Pediatr Gastroenterol Nutr* **60**, 433-40 (2015).
443. Lebherz, C. et al. Gene therapy with novel adeno-associated virus vectors substantially diminishes atherosclerosis in a murine model of familial hypercholesterolemia. *The Journal of Gene Medicine: A cross-disciplinary journal for research on the science of gene transfer and its clinical applications* **6**, 663-672 (2004).
444. Serrano-Mendioroz, I. et al. An inducible promoter responsive to different porphyrinogenic stimuli improves gene therapy vectors for acute intermittent porphyria. *Human gene therapy* **29**, 480-491 (2018).
445. Unzu, C. et al. Sustained enzymatic correction by rAAV-mediated liver gene therapy protects against induced motor neuropathy in acute porphyria mice. *Molecular Therapy* **19**, 243-250 (2011).
446. Yasuda, M. et al. AAV8-mediated gene therapy prevents induced biochemical attacks of acute intermittent porphyria and improves neuromotor function. *Molecular Therapy* **18**, 17-22 (2010).
447. Paneda, A. et al. Safety and liver transduction efficacy of rAAV5-cohPBGD in nonhuman primates: a potential therapy for acute intermittent porphyria. *Human gene therapy* **24**, 1007-1017 (2013).
448. Salido, E. et al. Phenotypic correction of a mouse model for primary hyperoxaluria with adeno-associated virus gene transfer. *Molecular Therapy* **19**, 870-875 (2011).
449. Murillo, O. et al. Liver Expression of a MiniATP7B Gene Results in Long-Term Restoration of Copper Homeostasis in a Wilson Disease Model in Mice. *Hepatology* **70**, 108-126 (2019).
450. Greig, J.A. et al. A gene therapy approach to improve copper metabolism and prevent liver damage in a mouse model of Wilson disease. *Human Gene Therapy Clinical Development* **30**, 29-39 (2019).
451. Murillo, O. et al. Long-term metabolic correction of Wilson's disease in a murine model by gene therapy. *Journal of hepatology* **64**, 419-426 (2016).
452. Chandler, R.J. & Venditti, C.P. Pre-clinical efficacy and dosing of an AAV8 vector expressing human methylmalonyl-CoA mutase in a murine model of methylmalonic acidemia (MMA). *Molecular genetics and metabolism* **107**, 617-619 (2012).
453. Guenzel, A.J., Collard, R., Kraus, J.P., Matern, D. & Barry, M.A. Long-term sex-biased correction of circulating propionic acidemia disease markers by adeno-associated virus vectors. *Human gene therapy* **26**, 153-160 (2015).
454. Guenzel, A.J., Hillestad, M.L., Matern, D. & Barry, M.A. Effects of adeno-associated virus serotype and tissue-specific expression on circulating biomarkers of propionic acidemia. *Human gene therapy* **25**, 837-843 (2014).
455. Chandler, R.J. et al. Adeno-associated virus serotype 8 gene transfer rescues a neonatal lethal murine model of propionic acidemia. *Human gene therapy* **22**, 477-481 (2011).
456. Yagi, H. et al. Complete restoration of phenylalanine oxidation in phenylketonuria mouse by a self-complementary adeno-associated virus vector. *The journal of gene medicine* **13**, 114-122 (2011).
457. Jung, S.-C. et al. Protective effect of recombinant adeno-associated virus 2/8-mediated gene therapy from the maternal hyperphenylalaninemia in offsprings of a mouse model of phenylketonuria. *Journal of Korean medical science* **23**, 877-883 (2008).
458. Mochizuki, S. et al. Long-term correction of hyperphenylalaninemia by AAV-mediated gene transfer leads to behavioral recovery in phenylketonuria mice. *Gene therapy* **11**, 1081-1086 (2004).
459. Ding, Z., Georgiev, P. & Thöny, B. Administration-route and gender-independent long-term therapeutic correction of phenylketonuria (PKU) in a mouse model by recombinant adeno-associated virus 8 pseudotyped vector-mediated gene transfer. *Gene therapy* **13**, 587-593 (2006).
460. Paulk, N.K. et al. Adeno-associated virus gene repair corrects a mouse model of hereditary tyrosinemia in vivo. *Hepatology* **51**, 1200-1208 (2010).
461. Chen, S.J., Tazelaar, J., Moscioni, A.D. & Wilson, J.M. In vivo selection of hepatocytes transduced with adeno-associated viral vectors. *Molecular Therapy* **1**, 414-422 (2000).
462. Aronson, S.J. et al. Liver-directed gene therapy results in long-term correction of progressive familial intrahepatic cholestasis type 3 in mice. *Journal of hepatology* **71**, 153-162 (2019).
463. Collaud, F. et al. Preclinical Development of an AAV8-hUGT1A1 Vector for the Treatment of Crigler-Najjar Syndrome. *Mol Ther Methods Clin Dev* **12**, 157-174 (2019).

464. Greig, J.A. et al. Determining the minimally effective dose of a clinical candidate AAV vector in a mouse model of Crigler-Najjar syndrome. *Molecular Therapy-Methods & Clinical Development* **10**, 237-244 (2018).
465. Bockor, L. et al. Repeated AAV-mediated gene transfer by serotype switching enables long-lasting therapeutic levels of hUgt1a1 enzyme in a mouse model of Crigler-Najjar Syndrome Type I. *Gene Ther* **24**, 649-660 (2017).
466. Ronzitti, G. et al. A translationally optimized AAV-UGT1A1 vector drives safe and long-lasting correction of Crigler-Najjar syndrome. *Molecular Therapy — Methods & Clinical Development* **3**, 16049 (2016).
467. Bortolussi, G. et al. Rescue of bilirubin-induced neonatal lethality in a mouse model of Crigler-Najjar syndrome type I by AAV9-mediated gene transfer. *FASEB J* **26**, 1052-63 (2012).
468. Pastore, N. et al. Sustained reduction of hyperbilirubinemia in Gunn rats after adeno-associated virus-mediated gene transfer of bilirubin UDP-glucuronosyltransferase isozyme 1A1 to skeletal muscle. *Hum Gene Ther* **23**, 1082-9 (2012).
469. Seppen, J. et al. Adeno-associated virus vector serotypes mediate sustained correction of bilirubin UDP glucuronosyltransferase deficiency in rats. *Mol Ther* **13**, 1085-92 (2006).
470. Demaster, A. et al. Long-term efficacy following readministration of an adeno-associated virus vector in dogs with glycogen storage disease type Ia. *Human gene therapy* **23**, 407-418 (2012).
471. Yiu, W.H. et al. Complete normalization of hepatic G6PC deficiency in murine glycogen storage disease type Ia using gene therapy. *Molecular Therapy* **18**, 1076-1084 (2010).
472. Koeberl, D.D. et al. AAV vector-mediated reversal of hypoglycemia in canine and murine glycogen storage disease type Ia. *Molecular Therapy* **16**, 665-672 (2008).
473. Ghosh, A. et al. Long-term correction of murine glycogen storage disease type Ia by recombinant adeno-associated virus-1-mediated gene transfer. *Gene therapy* **13**, 321-329 (2006).
474. Beaty, R. et al. Delivery of glucose-6-phosphatase in a canine model for glycogen storage disease, type Ia, with adeno-associated virus (AAV) vectors. *Gene Therapy* **9**, 1015-1022 (2002).
475. Wang, L. et al. AAV gene therapy corrects OTC deficiency and prevents liver fibrosis in aged OTC-knock out heterozygous mice. *Molecular genetics and metabolism* **120**, 299-305 (2017).
476. Hu, C. et al. Minimal ureagenesis is necessary for survival in the murine model of hyperargininemia treated by AAV-based gene therapy. *Gene therapy* **22**, 111-115 (2015).
477. Chandler, R.J. et al. Liver-directed adeno-associated virus serotype 8 gene transfer rescues a lethal murine model of citrullinemia type 1. *Gene therapy* **20**, 1188-1191 (2013).
478. Cunningham, S., Kok, C., Spinoulas, A., Carpenter, K. & Alexander, I. AAV-encoded OTC activity persisting to adulthood following delivery to newborn spf ash mice is insufficient to prevent shRNA-induced hyperammonaemia. *Gene therapy* **20**, 1184-1187 (2013).
479. Kasten, J. et al. Lethal phenotype in conditional late-onset arginase 1 deficiency in the mouse. *Molecular genetics and metabolism* **110**, 222-230 (2013).
480. Kok, C.Y. et al. Adeno-associated virus-mediated rescue of neonatal lethality in argininosuccinate synthetase-deficient mice. *Molecular Therapy* **21**, 1823-1831 (2013).
481. Wang, L. et al. Sustained correction of OTC deficiency in spf ash mice using optimized self-complementary AAV2/8 vectors. *Gene therapy* **19**, 404-410 (2012).
482. Cunningham, S.C. et al. Induction and prevention of severe hyperammonemia in the spfash mouse model of ornithine transcarbamylase deficiency using shRNA and rAAV-mediated gene delivery. *Molecular Therapy* **19**, 854-859 (2011).
483. Cunningham, S.C. et al. AAV2/8-mediated correction of OTC deficiency is robust in adult but not neonatal Spfash mice. *Molecular therapy* **17**, 1340-1346 (2009).
484. High, K.A. & Anguela, X.M. Adeno-associated viral vectors for the treatment of hemophilia. *Human Molecular Genetics* **25**, R36-R41 (2016).
485. Kattenhorn, L.M. et al. Adeno-associated virus gene therapy for liver disease. *Human gene therapy* **27**, 947-961 (2016).
486. Wang, D., Tai, P.W. & Gao, G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nature reviews Drug discovery* **18**, 358-378 (2019).
487. Berns, K.I. & Srivastava, A. Next generation of adeno-associated virus vectors for gene therapy for human liver diseases. *Gastroenterology Clinics* **48**, 319-330 (2019).
488. Büning, H. & Srivastava, A. Capsid modifications for targeting and improving the efficacy of AAV vectors. *Molecular Therapy-Methods & Clinical Development* **12**, 248-265 (2019).
489. D'Avola, D. et al. Phase I open label liver-directed gene therapy clinical trial for acute intermittent porphyria. *J Hepatol* (2016).
490. Mingozzi, F. et al. CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nat Med* **13**, 419-22 (2007).
491. Zabaleta, N., Hommel, M., Salas, D. & Gonzalez-Aseguinolaza, G. Genetic-Based Approaches to Inherited Metabolic Liver Diseases. *Human gene therapy* **30**, 1190-1203 (2019).

492. Diaz, G.A. et al. in Molecular therapy 2-3 (CELL PRESS 50 HAMPSHIRE ST, FLOOR 5, CAMBRIDGE, MA 02139 USA, 2019).
493. Weinstein, D.A., Ahmad, A., Lee, C., Poma, A. & Crombez, E. in MOLECULAR THERAPY 12-12 (CELL PRESS 50 HAMPSHIRE ST, FLOOR 5, CAMBRIDGE, MA 02139 USA, 2019).
494. Cotugno, G. et al. Long-term amelioration of feline Mucopolysaccharidosis VI after AAV-mediated liver gene transfer. *Molecular Therapy* **19**, 461-469 (2011).
495. Ferla, R. et al. Gene therapy for mucopolysaccharidosis type VI is effective in cats without pre-existing immunity to AAV8. *Human gene therapy* **24**, 163-169 (2013).
496. Cotugno, G. et al. Different serum enzyme levels are required to rescue the various systemic features of the mucopolysaccharidoses. *Human gene therapy* **21**, 555-569 (2010).
497. Ferla, R. et al. Similar therapeutic efficacy between a single administration of gene therapy and multiple administrations of recombinant enzyme in a mouse model of lysosomal storage disease. *Human gene therapy* **25**, 609-618 (2014).
498. Raben, N. et al. Replacing acid α-glucosidase in Pompe disease: recombinant and transgenic enzymes are equipotent, but neither completely clears glycogen from type II muscle fibers. *Molecular Therapy* **11**, 48-56 (2005).
499. Sun, B. et al. Efficacy of an adeno-associated virus 8-pseudotyped vector in glycogen storage disease type II. *Mol Ther* **11**, 57-65 (2005).
500. Wang, G. et al. Assessment of toxicity and biodistribution of recombinant AAV8 vector-mediated immunomodulatory gene therapy in mice with Pompe disease. *Mol Ther Methods Clin Dev* **1**, 14018 (2014).
501. Han, S.O. et al. Low-Dose Liver-Targeted Gene Therapy for Pompe Disease Enhances Therapeutic Efficacy of ERT via Immune Tolerance Induction. *Mol Ther Methods Clin Dev* **4**, 126-136 (2017).
502. Bortolussi, G. et al. Age-dependent pattern of cerebellar susceptibility to bilirubin neurotoxicity in vivo in mice. *Dis Model Mech* **7**, 1057-68 (2014).
503. Russell, D.W. & Hirata, R.K. Human gene targeting by viral vectors. *Nature genetics* **18**, 325-330 (1998).
504. Sedivy, J.M. & Sharp, P.A. Positive genetic selection for gene disruption in mammalian cells by homologous recombination. *Proceedings of the National Academy of Sciences* **86**, 227-231 (1989).
505. Miller, D.G. et al. Gene targeting in vivo by adeno-associated virus vectors. *Nature biotechnology* **24**, 1022-1026 (2006).
506. Pankowicz, F.P. et al. Reprogramming metabolic pathways in vivo with CRISPR/Cas9 genome editing to treat hereditary tyrosinaemia. *Nature communications* **7**, 1-6 (2016).
507. Overturf, K. et al. Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinaemia type I. *Nature genetics* **12**, 266-273 (1996).
508. Borel, F. et al. Survival advantage of both human hepatocyte xenografts and genome-edited hepatocytes for treatment of α-1 antitrypsin deficiency. *Molecular Therapy* **25**, 2477-2489 (2017).
509. Haapaniemi, E., Botla, S., Persson, J., Schmierer, B. & Taipale, J. CRISPR–Cas9 genome editing induces a p53-mediated DNA damage response. *Nature medicine* **24**, 927-930 (2018).
510. Ihry, R.J. et al. p53 inhibits CRISPR–Cas9 engineering in human pluripotent stem cells. *Nature medicine* **24**, 939-946 (2018).
511. Charlesworth, C.T. et al. Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nature medicine* **25**, 249-254 (2019).
512. Kotin, R.M., Linden, R.M. & Berns, K.I. Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. *The EMBO journal* **11**, 5071-5078 (1992).
513. Connelly, J.P., Barker, J.C., Pruett-Miller, S. & Porteus, M.H. Gene correction by homologous recombination with zinc finger nucleases in primary cells from a mouse model of a generic recessive genetic disease. *Molecular therapy* **18**, 1103-1110 (2010).
514. DeKelver, R.C. et al. Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome. *Genome research* **20**, 1133-1142 (2010).
515. Hockemeyer, D. et al. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nature biotechnology* **27**, 851-857 (2009).
516. Ou, L. et al. ZFN-mediated in vivo genome editing corrects murine Hurler syndrome. *Molecular Therapy* **27**, 178-187 (2019).
517. Barzel, A. et al. Promoterless gene targeting without nucleases ameliorates haemophilia B in mice. *Nature* **517**, 360-364 (2015).
518. Porro, F. et al. Promoterless gene targeting without nucleases rescues lethality of a Crigler-Najjar syndrome mouse model. *EMBO molecular medicine* **9**, 1346-1355 (2017).
519. Smith, L.J. et al. Stem cell-derived clade F AAVs mediate high-efficiency homologous recombination-based genome editing. *Proceedings of the National Academy of Sciences* **115**, E7379-E7388 (2018).

520. Wright, J. et al. in Molecular therapy 462-463 (CELL PRESS 50 HAMPSHIRE ST, FLOOR 5, CAMBRIDGE, MA 02139 USA, 2019).
521. Thakore, P.I. et al. RNA-guided transcriptional silencing in vivo with S. aureus CRISPR-Cas9 repressors. *Nature communications* **9**, 1-9 (2018).
522. Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A. & Liu, D.R. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* **533**, 420-424 (2016).
523. Villiger, L. et al. Treatment of a metabolic liver disease by in vivo genome base editing in adult mice. *Nature medicine* **24**, 1519-1525 (2018).
524. Mangeot, P.E. et al. Genome editing in primary cells and in vivo using viral-derived Nanoblades loaded with Cas9-sgRNA ribonucleoproteins. *Nature communications* **10**, 1-15 (2019).
525. Schuh, R.S. et al. In vivo genome editing of mucopolysaccharidosis I mice using the CRISPR/Cas9 system. *Journal of controlled release* **288**, 23-33 (2018).
526. Finn, J.D. et al. A single administration of CRISPR/Cas9 lipid nanoparticles achieves robust and persistent in vivo genome editing. *Cell reports* **22**, 2227-2235 (2018).
527. Landau, D.J. et al. 311. In Vivo Zinc Finger Nuclease-Mediated Targeted Integration of a Glucose-6-Phosphatase Transgene Enhances Biochemical Correction in Mice with Glycogen Storage Disease Type IA. *Molecular Therapy* **24**, S125 (2016).
528. Sarcar, S. et al. Next-generation muscle-directed gene therapy by in silico vector design. *Nature communications* **10**, 1-16 (2019).
529. Mingozzi, F. & High, K.A. Overcoming the host immune response to adeno-associated virus gene delivery vectors: the race between clearance, tolerance, neutralization, and escape. *Annual review of virology* **4**, 511-534 (2017).
530. Prasad, S. et al. in HUMAN GENE THERAPY A125-A125 (MARY ANN LIEBERT, INC 140 HUGUENOT STREET, 3RD FL, NEW ROCHELLE, NY 10801 USA, 2018).
531. Magami, Y. et al. Cell proliferation and renewal of normal hepatocytes and bile duct cells in adult mouse liver. *Liver* **22**, 419-425 (2002).
532. Malato, Y. et al. Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. *The Journal of clinical investigation* **121**, 4850-4860 (2011).
533. Lotfinia, M., Abdollahpour-Alitappeh, M., Hatami, B., Zali, M.R. & Karimipoor, M. Adeno-associated virus as a gene therapy vector: strategies to neutralize the neutralizing antibodies. *Clinical and experimental medicine*, 1-10 (2019).
534. Vandamme, C., Adjali, O. & Mingozzi, F. Unraveling the complex story of immune responses to AAV vectors trial after trial. *Human gene therapy* **28**, 1061-1074 (2017).
535. Tse, L.V., Moller-Tank, S. & Asokan, A. Strategies to circumvent humoral immunity to adeno-associated viral vectors. *Expert opinion on biological therapy* **15**, 845-855 (2015).
536. Masat, E., Pavani, G. & Mingozzi, F. Humoral immunity to AAV vectors in gene therapy: challenges and potential solutions. *Discov Med* **15**, 379-89 (2013).
537. Majowicz, A. et al. Successful repeated hepatic gene delivery in mice and non-human primates achieved by sequential administration of AAV5ch and AAV1. *Molecular Therapy* **25**, 1831-1842 (2017).
538. Pan, D., Büning, H. & Ling, C. Rational design of gene therapy vectors. *Molecular Therapy-Methods & Clinical Development* **12**, 246-247 (2019).
539. Paulk, N.K. et al. Bioengineered AAV capsids with combined high human liver transduction in vivo and unique humoral seroreactivity. *Molecular Therapy* **26**, 289-303 (2018).
540. Herrmann, A.-K. et al. A robust and all-inclusive pipeline for shuffling of adeno-associated viruses. *ACS synthetic biology* **8**, 194-206 (2018).
541. Yao, T. et al. Site-specific PEGylated adeno-associated viruses with increased serum stability and reduced immunogenicity. *Molecules* **22**, 1155 (2017).
542. Meliani, A. et al. Enhanced liver gene transfer and evasion of preexisting humoral immunity with exosome-enveloped AAV vectors. *Blood Adv* **1**, 2019-2031 (2017).
543. Orefice, N.S. et al. Real-time monitoring of exosome enveloped-AAV spreading by endomicroscopy approach: a new tool for gene delivery in the brain. *Molecular Therapy-Methods & Clinical Development* **14**, 237-251 (2019).
544. Pien, G.C. et al. Capsid antigen presentation flags human hepatocytes for destruction after transduction by adeno-associated viral vectors. *The Journal of clinical investigation* **119**, 1688-1695 (2009).
545. Townsend, M.J., Monroe, J.G. & Chan, A.C. B-cell targeted therapies in human autoimmune diseases: an updated perspective. *Immunological reviews* **237**, 264-283 (2010).
546. Meliani, A. et al. Antigen-selective modulation of AAV immunogenicity with tolerogenic rapamycin nanoparticles enables successful vector re-administration. *Nat Commun* **9**, 4098 (2018).
547. Kishimoto, T.K. Development of ImmTOR Tolerogenic Nanoparticles for the Mitigation of Anti-drug Antibodies. *Frontiers in Immunology* **11** (2020).

548. Jordan, S.C. et al. IgG endopeptidase in highly sensitized patients undergoing transplantation. *New England Journal of Medicine* **377**, 442-453 (2017).
549. Leborgne, C. et al. IgG-cleaving endopeptidase enables in vivo gene therapy in the presence of anti-AAV neutralizing antibodies. *Nature Medicine*, 1-6 (2020).
550. Chicoine, L.G. et al. Plasmapheresis eliminates the negative impact of AAV antibodies on microdystrophin gene expression following vascular delivery. *Molecular Therapy* **22**, 338-347 (2014).
551. Monteilhet, V. et al. A 10 patient case report on the impact of plasmapheresis upon neutralizing factors against adeno-associated virus (AAV) types 1, 2, 6, and 8. *Mol Ther* **19**, 2084-91 (2011).
552. Bertin, B. et al. Capsid-specific removal of circulating antibodies to adeno-associated virus vectors. *Scientific reports* **10**, 1-11 (2020).



Part I

NEXT GENERATION APPROACHES FOR POMPE DISEASE TREATMENT

Chapter 2

Progress and challenges for gene therapy in Pompe disease

Modified from
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1. Introduction

1.1 Pompe Disease

Pompe disease (PD, OMIM #232300) is an autosomal recessive disorder due to mutations in the gene coding for acid alpha-glucosidase (GAA). GAA is the enzyme that degrades lysosomal glycogen to free glucose. Mutations that decrease the activity of this enzyme lead to the accumulation of glycogen in the lysosomal compartment and to the impairment of lysosomal function and autophagy flux.[1] In PD, glycogen accumulates virtually in all tissues but the disease manifestation is prominently muscular, with severe hypertrophic cardiomyopathy, respiratory function impairment and proximal muscle weakness.[2] Accumulation of lysosomal glycogen in the central nervous sytem (CNS) is associated with white matter abnormalities and cognitive impairment in particular in patients with no residual GAA activity.[3] Additional pathological changes associated with the disease have also been described, i.e., cerebral vessels abnormalities that are relatively frequently found in adult Pompe patients.[4] The disease can be broadly classified into i) infantile-onset PD (IOPD), with no residual GAA activity and associated with generalized hypotonia, and cardio-respiratory failure, leading to death in the first year of life; and ii) late-onset PD (LOPD), characterized by residual levels of GAA activity and a less severe phenotype with progressive limb muscle weakness and respiratory insufficiency.[5] PD is the first and probably the more comprehensively characterized LSD that manifests as a metabolic myopathy. For this reason, over the course of the last few decades, several therapeutic approaches aimed at addressing the pathology were explored.

1.2 Pompe Disease mouse models

Natural GAA deficiency has been reported in large animals such as cats, dogs, sheep or cattle.[6] However, in order to facilitate laboratory research, several murine models have been generated by disrupting different exons on the *Gaa* gene. The availability of these *Gaa* knock-out (KO) mice, has been crucial to study and characterize the disease pathophysiology, the underlying molecular mechanisms, disease biomarkers and to test therapeutic approaches.[7, 8] Although all these models should reproduce the IOPD phenotype, the disease severity is greatly modulated by the genetic backgrounds in which the different models have been created.

The most severe and used *Gaa* KO mouse model was generated in 1998 by Raben and colleagues in a mixed C57BL/6;129 background ($Gaa^{6neo/6neoB6;129\text{-}Gaatm1Rabn/J}$)[7] and recapitulates key PD features like the whole body glycogen accumulation,[7, 9] impaired autophagic flux in skeletal muscle,[10-12] neuropathology,[13-15] cardiac defects[16] and muscle weakness.[7, 11] However, despite complete GAA deficiency and systemic glycogen accumulation, this 6*neo*/6*neo* model exhibited a mild phenotype with intermediate features between the early and late onset disease forms.[7, 9, 12, 15, 16] In particular, the lack of important respiratory defects represents a key limitation of this mouse as model for PD.[11, 15, 16] Recently, by exploiting the DBA2/J genetic background and in particular, the *Latent TGF-β-binding protein 4* (*Ltbp4*) modifier gene,[17-21] we generated a novel *Gaa* KO mouse model that recapitulates the main key disease manifestations in humans, including severe respiratory impairment and neuroglial spinal cord defects at early age.[22]

Immunogenicity of recombinant human acid alpha-glucosidase (rhGAA), the current standard of care for PD, in *Gaa* KO mice is a limitation to study the long-term efficacy of enzyme replacement therapy (ERT) *in vivo* and to perform a side-by-side comparison with

gene transfer.[16, 23] To overcome this constraint, we generated a CD4-deficient PD mouse model (paper in revision), unable to mount humoral immunity towards protein replacement therapies,[24, 25] but presenting the same disease features than the immunocompetent 6*neo*/6*neo* model.[7]

2. Standard of care and next generation enzyme replacement therapy (ERT)

The discovery of the pathway of uptake of lysosomal enzymes mediated by M6P receptor allowed the introduction of the concept of cross-correction, i.e., the possibility to replace a lysosomal enzyme by supplementing the enzyme in the extracellular media.[26-28]

The development of the ERT considerably changed the prognosis of PD and this treatment modality represents the current standard of care for the disease. Indeed, pioneering clinical studies in IOPD patients clearly demonstrated the efficacy of the ERT for the improvement of the cardiac and muscle function.[29-31] These results led to the approval in 2006 of the first therapy for IOPD followed in 2010 by the approval of ERT for LOPD (alglucosidase alfa, Lumizyme® within the USA and Myozyme® outside of the USA; Sanofi Genzyme). RhGAA is administered intravenously every other week at a recommended dose of 20 mg/kg, but higher dose regimens (up to 40 mg/kg) are also administered in IOPD patients. These doses are markedly higher than those required in other lysosomal storage disorders, possibly reflecting the higher threshold for correction of GAA deficiency in the skeletal muscle of Pompe patients. In addition, the liver takes up most of the rhGAA (up to 85%) and considerably limits muscle targeting. ERT with recombinant human GAA has clearly demonstrated cardiac and respiratory function improvement and has significantly extended the lifespan of IOPD patients.[32] However, the administration of rhGAA in IOPD is frequently associated with the development of neutralizing humoral immune responses against the enzyme and decreased treatment efficacy and survival. This is particularly relevant for cross-reactive immune-material negative (CRIM–) patients with no residual GAA antigen, who therefore completely lack central tolerance to the protein.[33] Another important shortcoming of ERT is related to its limited efficacy, for example, respiratory function is only partially rescued by the treatment, and approximately 30% of the rhGAA-treated patients end up requiring assisted ventilation, either invasive or not, over the course of their life.[34] Skeletal muscle function is also improved by ERT, although the effect of the treatment is quite variable with some subjects maintaining independent ambulation and others showing only minor improvements and eventually ending up being wheelchair bound.[35, 36] The majority of reports to date of clinical trials or investigator-initiated clinical studies in LOPD patients are concordant in concluding that, in most patients, ERT leads to an improvement of muscle function as measured by 6-minute walk test whereas long-term studies show that respiratory function is only stabilized.[37] Available studies also show that ERT may stabilize or even slightly improve muscle strength and respiratory function among patients at advanced stages of the disease.[38, 39] In addition to the efficacy limitations, i) the requirement for frequent intravenous infusions of high doses of rhGAA, ii) the possibility of severe and detrimental immune responses in both CRIM− and CRIM+ patients[29, 36, 40] and iii) the inability of the recombinant enzyme to cross BBB and correct the nervous system,[41] make the development of new therapies for PD an urgent need.

Since the development of ERT for PD, efforts were dedicated to overcoming some of the limitations of the treatment. Two main strategies, both aimed at the enhancement of the enzyme bioavailability in tissues, are now in late-stage clinical testing. The first approach

consists in the modification of the recombinant enzyme to increase the M6P residue content[42] ; the second strategy involves the use of pharmacological chaperones (PCs) to enhance ERT efficacy.[43, 44]

A second generation ERT with rhGAA with higher affinity for the M6P receptors is now under evaluation in a phase III clinical trial (avalglucosidase alfa, Neo-GAA; Sanofi Genzyme; NCT02782741).[45] The study is designed to test doses ranging from 5 to 20 mg/kg of biweekly-administered Neo-GAA with the possibility to switch doses during the study. This phase III clinical trial, aimed at the comparison of the efficacy and safety of bi-weekly infusions of avalglucosidase alfa and alglucosidase alfa in patients with LOPD, is still recruiting. Another experimental rhGAA called ATB200 (Amicus Therapeutics), with a higher content of M6P and bis-M6P glycan residues was developed and is being tested in a clinical trial in association with pharmacological chaperones (vide infra). In preclinical studies, GAA enzymes engineered with synthetic M6P residues improved muscle function in Pompe mice either alone or in combination with PCs and showed enhanced targeting in Pompe patients fibroblasts when compared to first-generation rhGAA.[42, 46, 47]

Another strategy tested to improve bioavailability of the GAA enzyme in tissues, thus resulting in enhanced clearance of glycogen, consists in the use of uptake domains. Several chimeric GAA proteins carrying uptake domains were tested in preclinical animal models of PD.[48-50] Among these, an engineered form of rhGAA carrying the glycosylation-independent lysosomal targeting (GILT) domain for tissue uptake[49-51] was tested in LOPD patients and reached late-stage clinical development (NCT01924845, BMN 701, BioMarin Pharmaceutical). Unfortunately, concerns over the development of hypoglycemia following enzyme infusion resulted in the discontinuation of the development.[52] More recently, a chimeric form of rhGAA containing a humanized Fab fragment derived from the murine 3E10 antibody also entered phase I/II clinical testing (NCT02898753, VAL1221, Valerion Therapeutics, LLC).[50]

The use of pharmacological agents is emerging as a potential strategy to improve the efficacy of ERT in PD. The combination of rhGAA with β2 agonists, e.g., clenbuterol or albuterol mediated increased M6P receptor expression, improved muscle function and reduced glycogen accumulation in muscle and brain when the enzyme was supplied by ERT or by liver-mediated gene therapy (vide infra).[53-55] A phase I/II clinical trial for the evaluation of the combination of albuterol and rhGAA showed increased M6P receptor expression in muscle biopsies and motor function improvement.[56] A second clinical trial on the effects of clenbuterol on the ERT efficacy showed improved motor function and the correction of molecular biomarkers of the disease in muscle.[57] Both these early-phase trials, realized in LOPD patients, showed signs of improved efficacy in combination with ERT with only mild secondary effects. Larger trials will be required to clearly demonstrate the advantage of the approach compared to ERT alone.

Another combination approach for PD treatment is based on the use of pharmacological chaperone therapy (PCT). Chaperones are small molecules known to promote folding and improve stability of proteins and enzymes.[58] In the case of PD, improved rhGAA bioavailability via enhancement of enzyme stability in blood was demonstrated in preclinical models[43, 59]. Different glucose analogues, acting as allosteric inhibitors of GAA, have been investigated (e.g., 1-Deoxynojirimycin (1-DNJ; Duvoglustat®), 1-Deoxynojirimycin-HCl (DNJ-HCl; Duvoglustat®-HCl; AT2220) or N-butyl-deoxynojirimycin (NB-DNJ, Miglustat®)) and demonstrated a beneficial effect when combined with ERT.[60-62] Recent clinical studies sponsored by Amicus Therapeutics tested the combination of the AT2221

chaperone with the engineered enzyme ATB200 in LOPD patients (NCT02675465, AT-GAA, Amicus Therapeutics).

3. Gene therapy

Given its monogenic origin, PD represents an ideal target for the development of gene replacement strategies. Since 1998, year of the first in vivo gene therapy approach for PD[63], different in vivo and ex vivo approaches were tested in animal models with the aim of correcting the PD phenotype **(Figure 2.1)**.[64-67] These approaches are reviewed elsewhere, and this report will mainly focus on the discussion of recent advances in the field of in vivo gene therapy with adeno-associated virus (AAV) vectors.[8, 68]

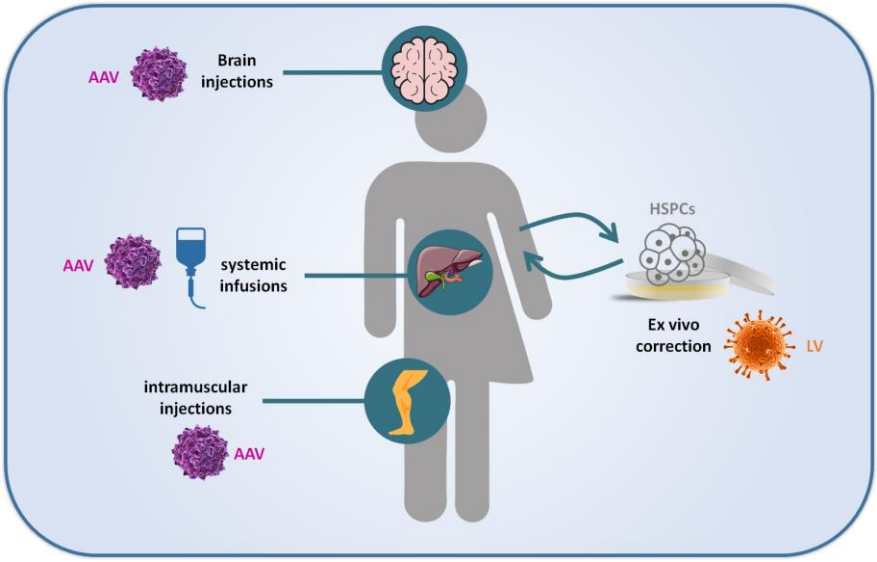

Figure 2.1 Gene therapy modalities for PD
In vivo gene therapy consists in the direct administration of a gene delivery vector (viral or non-viral) directly into the recipient of gene transfer. In Pompe disease, most of the experience to date comes from AAV vector-mediated gene transfer. AAV vectors have been administered either directly into the bloodstream to target the muscle[69], the liver[15, 70], or multiple tissues[71], or directly into muscle[72], or intracerebroventricular to target the central nervous system[73]. Ex vivo gene therapy uses autologous CD34+ hematopoietic progenitors transduced with integrative vectors (e.g., lentiviral vectors)[74] and re-infused in the recipient following myeloablative bone marrow conditioning. This gene therapy modality has been shown to have the potential to efficiently deliver GAA to the central nervous system. AAV, adeno-associated virus vectors; GAA, acid alpha-glucosidase.

Gene therapy holds the potential for improving the standard of care for PD, addressing some of the key limitations of ERT (
Table 2-1). Consistently, in recent years the landscape of gene therapy for PD has dramatically changed, with a pipeline of candidate therapeutics at various stages of development (**Table 2-2**).

Over the past two decades, the collective experiences across several research groups demonstrated the potential of AAV vectors encoding GAA to rescue PD in animal models, along with the potential for reducing GAA immunogenicity.[15, 72, 75-79] Different AAV serotypes have been explored to develop gene transfer strategies to treat PD in animal models, including AAV1[80-83], AAV2[72], AAV5[84, 85], AAV6[75], AAV8[15, 76, 85-87] and AAV9[14, 16, 73, 83, 88-90]. In these strategies, the muscle[16, 72, 75, 76, 80-82, 85, 88, 89], the liver[76, 86, 87], or the CNS[14, 73, 83, 90] were targeted.

Table 2-1 Comparison of enzyme replacement therapy with gene therapy for Pompe disease

Features	Enzyme replacement therapy	Investigational gene therapy
Safety and efficacy in patients	Safe and effective in Pompe patients; long-term efficacy achieved in a subset of patients; hypersensitivity reactions sometimes observed	Safety and efficacy not yet established in Pompe patients
GAA immunogenicity	Observed in both IOPD and LOPD patients; associated with treatment failures in IOPD patients	Potentially a concern for some gene therapy modalities targeting the muscle
Whole-body correction	Not achievable, rhGAA does not cross blood-brain barrier at the doses used	Potential for body-wide correction of the disease
Cross-correction	Feasible, highly dependent on levels of expression of mannose 6-phosphate receptor	Feasible ; potential for expressing GAA directly in target tissues
Immune tolerance induction	Achievable but difficult and requires co-administration of immunomodulatory drugs	Potentially achievable with liver gene transfer

Table 2-2 Biotechnology companies currently developing gene therapies for Pompe disease

Company	Gene therapy vector	Transgene	Target tissue	Development status
Actus (AskBio)#	AAV (in vivo)	GAA	Liver	Phase I/II
Audentes	AAV (in vivo)	GAA	Muscle and liver	Clinical trial-enabling
Sarepta*	AAV (in vivo)	GAA	Central nervous system	Preclinical
Spark**	AAV (in vivo)	Secretable GAA	Liver	Clinical trial-enabling
Amicus	AAV (in vivo)	Secretable GAA?	Liver	Preclinical
Regeneron	AAV (in vivo)	CD63-GAA fusion	Liver	Preclinical
AvroBio	Lentivirus (ex vivo)	GILT-GAA fusion	CD34+ HSC	Preclinical

*#, therapeutic candidate inlicensed from Duke University; *, therapeutic candidate inlicensed from Lacerta, Inc.; **, therapeutic candidate inlicensed from Genethon; HSC, hematopoietic stem cells; GILT, glycosylation-independent lysosomal targeting uptake domain; CD63, tetraspanin binding domain*

3.1 Intramuscular and systemic gene transfer

First evidences of the efficacy of AAV-based gene transfer efficacy in a PD mouse model were obtained by intramuscular injection of AAV vectors[72, 82, 83, 88]. This route of administration was associated with increased GAA transgene immunogenicity and correction of the glycogen accumulation only locally at the level of the injected muscle groups.[72] This initial work was followed by studies in which AAV vectors were given systemically or injected directly into the diaphragm. Intra-diaphragmatic injections of AAV vectors led to improvement in respiratory function in $Gaa^{-/-}$ mice. These studies also indicated the ability of AAV vectors to transduce efficiently the phrenic motor neurons that innervates the diaphragm.[78, 81, 83] Based on these results a first-in-human trial of gene therapy for PD with direct injection of an AAV vector expressing GAA in the diaphragm was initiated. This trial, conducted in IOPD patients who required assisted ventilation, established the safety profile of the approach.[91] Development of antibodies directed against both the AAV capsid and GAA were observed, in the absence of detectable T cell responses against the vector or the transgene, consistent with the fact that participants were immunosuppressed at the time of vector administration.[91] Given the limited size of the trial, no definitive conclusions could be drawn from this study in terms of therapeutic efficacy, aside from a trend toward improved respiratory function.[91-94]

Currently, the intramuscular delivery of an AAV9 vector expressing GAA is being tested in a phase I/II trial. In this study, the vector is given concomitantly with an immunosuppressive regimen based on sirolimus (rapamycin) and the B cell-depleting monoclonal antibody rituximab, in an effort to determine if the approach allows for two consecutive intramuscular administrations of AAV vectors while blocking humoral immune responses to the AAV capsid (NCT02240407).

While intramuscular administration of AAV vectors allows to achieve highly efficient and long term tissue transduction, concerns over transgene immunogenicity and the systemic nature of the disease, make this route of vector delivery unsuitable to treat effectively PD.[95, 96] The recent advances in the ability to produce AAV at large scale, and the exciting results of clinical trials of systemic delivery of AAV vector to treat neuromuscular diseases, resulted in several preclinical studies of systemic delivery of AAV vectors containing muscle-specific expression cassettes for the GAA transgene.[97] These studies demonstrated efficient clearance of glycogen accumulation in muscle and significant improvement of muscle strength as well as cardiac and respiratory function.[16, 72, 75, 76, 78, 80-82, 85, 88, 89]

However, one limitation of the approach is that muscle targeting via the systemic route requires extremely high doses of vector exceeding 10^{14} vector genome/kg, not easily achievable when addressing the LOPD patient population, [98-100] and associated with risks of toxicity.[101] Furthermore, muscle-specific expression of GAA is associated with an increased risk of anti-GAA antibody formation and potential immunotoxicities.[85, 102] One potential solution to the issue of transgene immunogenicity is the use of liver-muscle tandem promoters, although this approach is also more suitable for IOPD patients, due to the high vector doses required to transduce muscle in adults with AAV vectors delivered systemically.[71]

3.2 Liver gene therapy for PD

Liver gene transfer has been explored as a modality to treat PD. Early studies by Amalfitano and colleagues demonstrated that liver expression of GAA by adenoviral gene transfer mediated cross-correction in the skeletal muscle.[66] However, the expression of the transgene in circulation was only transient likely due to the induction of an immune response against the transgene elicited by the vector used in the study.

Stable expression of GAA in the liver was achieved via AAV vector-mediated gene transfer, resulting in cross-correction in peripheral organs with no evident immunogenicity against the transgene.[77, 103, 104]. Further studies confirmed the uptake of GAA in heart and skeletal muscle with glycogen clearance and improved muscle function.[15, 70, 105] Collectively, the studies of GAA gene transfer to the liver confirmed the potential efficacy profile and protolerogenic potential of this strategy and, together with toxicology studies in mice, supported the initiation of a phase I/II clinical trial sponsored by Actus Therapeutics (AskBio, now Bayer group) (NCT03533673, **Table 2-2**).[87] The primary objective of this trial is to demonstrate the safety of AAV vector-mediated liver gene transfer for GAA in LOPD patients. Secondary outcome measures aim at the characterization of the biodistribution of the enzyme produced by the liver and at the evaluation of the muscle glycogen accumulation and rescue of muscle and respiratory function.

A limitation of liver gene transfer for GAA is that the supply of the enzyme from the liver to peripheral tissues relies exclusively on cross-correction. Optimization of GAA secretion into the bloodstream therefore could represent an important step toward the development of a safe and efficacious liver-targeted gene therapy for PD. The use of a heterologous signal peptide to improve the secretion of GAA into the bloodstream was previously reported.[106] We further improved this strategy by using an optimized signal peptide and a truncated version of the GAA protein with enhanced secretion a modification determined using bioinformatics prediction.[107] Using this second generation engineered secretable GAA transgene, we demonstrated a clear dose advantage when compared to the native version of

the GAA transgene expressed in the liver.[15] In particular, sustained circulating levels of the enzyme resulted in long-term correction of glycogen accumulation in tissues known to be refractory for GAA enzyme uptake (e.g., triceps and quadriceps), or tissue separated from the systemic circulation by physical barriers spinal cord and brain, which are shielded from most circulating proteins by the blood-brain barrier (BBB) (**Figure 2.2**).

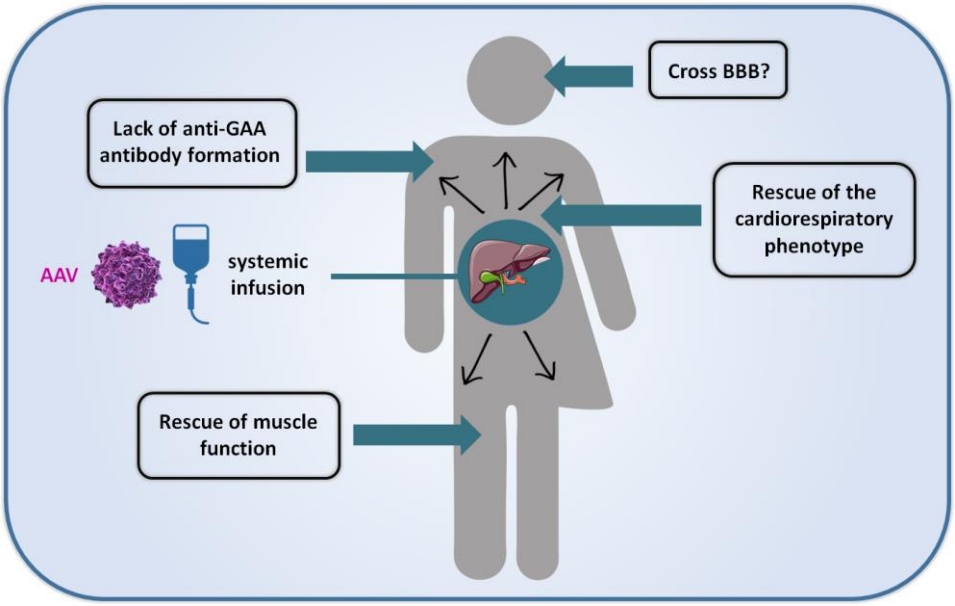

Figure 2.2 Investigational liver-directed gene therapy for Pompe disease
The graph exemplifies the potential for gene therapy as observed in preclinical studies in Gaa$^{-/-}$ mice (45). Targeting of the liver with AAV vectors expressing secretable forms of GAA can potentially transform the organ in a bio factory of GAA enzyme delivered to the entire body. Experiments in Gaa$^{-/-}$ mice show that the expression of secretable GAA results in the rescue of muscle and cardiorespiratory impairment with reduced GAA transgene immunogenicity. Partial correction of glycogen accumulation in the central nervous system is also observed. BBB, blood-brain barrier; GAA, acid alpha-glucosidase.

We also reported efficient rescue of muscle strength impairment and respiratory function.[12, 15, 22] Interestingly, the use of secretable GAA also resulted in a better control of anti-transgene humoral immune responses, possibly via the efficient induction of regulatory T cells in draining lymph nodes (**Figure 2.2**).[108] Scale-up of this approach in non-human primates demonstrated the uptake of the enzyme secreted by the liver in peripheral tissues, particularly the heart without associated toxicity and supported the translation of this approach into the clinic (paper in revision). Spark Therapeutics is currently engaged in the clinical translation of these proof-of-concept results (**Table 2-2**).

Another potential limitation of liver gene transfer for PD is related to the fact that hepatic gene transfer with AAV vectors in neonate animals does not persist at long-term.[109] This poses important challenges to the use of this therapeutic strategy in IOPD patients. As previously mentioned (**Chapter 1**) in pediatric patients, liver growth is likely to decrease the treatment efficacy due to vector genome dilution, potentially resulting in progressive loss of transgene expression. The formation of a NAbs after AAV vector administration would precludes any further administration of the vector. Ongoing work aimed at the identification of immunosuppressive treatments that would allow for vector re-administration yielded promising preclinical results, and some of them has been translated to the clinic (NCT02240407, University of Florida).[110-112]

A possible alternative to vector re-administration is based on the use of tandem promoters, which would allow for the induction of GAA transgene immune tolerance through liver expression, while driving persistent transgene expression in non-hepatic tissues.[71] This strategy could potentially represent a path forward toward the development of a safe and long-lasting gene transfer approach for IOPD.

3.3 Other gene therapy approaches to PD

CNS-targeted delivery of AAV vectors expressing GAA has been recently explored as a possible therapeutic strategy for IOPD, based on the growing evidence that the CNS is significantly affected by glycogen accumulation in this patient population. Results obtained to date demonstrated that the clearance of glycogen in the CNS resulted in the rescue of the functional impairment associated with PD.[73, 113] However, restricted targeting of CNS is associated with a limited correction of the muscle defect.[84, 114, 115] One possible solution around this is the use of tandem liver-neuron promoters driving simultaneous expression of GAA in liver and CNS after systemic administration of AAV vectors able to cross the BBB.[71]

Gene replacement therapy with the GAA transgene is the obvious approach to PD. Nevertheless, given the complexity of the disease, development of alternative therapeutic strategies might result in enhanced efficacy or in the development of adjuvant therapies to be combined with protein- or gene-replacement approaches. Overexpression of the transcription factor EB (TFEB) has been explored as a possible avenue to treat PD.[116] In $Gaa^{-/-}$ mice, the administration of AAV vectors encoding for TFEB in muscle were shown to induce lysosomal exocytosis that was associated with improved muscle performance and delayed disease progression in the absence of reduction of glycogen content in skeletal muscle.[117]

SRT has also been proposed for PD.[118-120] This strategy is based on the reduction of the activity of the glycogen synthase enzyme acting in muscle (GYS1) to reduce the accumulation of glycogen. Accordingly, using transgenic mouse models, Douillard-Guilloux and colleagues demonstrated that the genetic suppression of the muscle-specific glycogen synthase in $Gaa^{-/-}$ mice was able to reverse cardiac impairment, reduce glycogen accumulation and improve muscle strength[119]. Another study, designed to inhibit GYS1 using a phosphorodiamidate morpholino oligonucleotide (PMO) conjugated to a cell penetrating peptide (GS-PPMO), showed decreased glycogen accumulation in heart and skeletal muscle of Pompe mice.[118] One caveat related to this approach is that excessive knockdown of GYS1 may lead to toxicities similar to those observed in GSD type 0, which is characterized by an increased risk of cardiac arrest.[121]

Another approach based on the use of PMO to promote exon inclusion and correct the common mutation c.-32-13T>G has also been proposed.[122] Tricyclo-DNA antisense oligonucleotides were also tested as a strategy to correct the aberrant splicing mutation commonly found in LOPD patients.[123]

4. Gene therapy vs. ERT

An intriguing finding coming from the preclinical studies with secretable GAA, and likely to be generalizable to all gene therapies, is the time-dependent clearance of glycogen in tissues.[15] This is likely driven by the steady-state, continuous exposure to the GAA enzyme expressed as a transgene as opposed to the administration of the recombinant form of the

enzyme in the setting of ERT, which drives a transient increase in enzyme activity in peripheral tissues. This may provide an advantage for gene therapy, compared to the peak and trough kinetics observed after ERT administration. Notably, results from studies with rhGAA given in combination with pharmacological chaperones support this concept, as the enhanced half-life of GAA mediated by the chaperone molecule results in improved efficacy.[42-44] To this aim, gene therapy, when successful in driving sustained levels of GAA expression and uptake into peripheral tissues, is likely to be superior to any ERT, owing its unique pharmacokinetics profile.

5. Concluding remarks

PD is a debilitating and potentially fatal disease. The development of ERT for the disease, more than a decade ago represented a breakthrough in the management of the disease, particularly for IOPD patients.[5, 30, 32] Today, PD remains an unmet medical need, as immunogenicity of recombinant GAA and long-term outcomes of ERT point out to the need for better treatments, both for pediatric and adult patients. Next generation ERTs are in the pipeline, however, because they mostly rely on the same mechanism of action of the current ERT, they are likely to result in only incremental benefit for patients.[48-50]

Gene therapy holds the potential to revolutionize the way we treat PD, virtually providing a steady state supply of GAA enzyme to the entire body following a single medical intervention. Promising results obtained in preclinical studies in animal models of PD, along with results from clinical trials for various monogenic diseases, generated a lot of excitement about the prospect of a gene therapy for PD. As for any new investigational therapy, the primary goal of these early gene therapy trials should be focused on safety and on the potential limitations of the current gene transfer technologies.[124, 125]

Additionally, PD is extremely challenging and diverse, with IOPD patients presenting with clinical features quite different from those in the adult LOPD patient population. Furthermore, even within a category of patients, and even in patients carrying the same GAA genetic background (like most of LOPD patients), inter patient variability is extremely high, making trial design complex, also in view of the fact that endpoints of efficacy have not evolved significantly since the approval of ERT for PD and are far from being sensitive.

These complexities constitute important disease-specific challenges that will shape the future development of gene-based approaches for PD. Given the different mechanism of action of investigational gene therapy vs. ERT, future exploratory clinical work will likely help gaining a better understanding of whether the current measures of clinical outcomes used in ERT are best suited to capture the potential benefit of gene therapy.

6. References

1. Raben, N. et al. Replacing acid α-glucosidase in Pompe disease: recombinant and transgenic enzymes are equipotent, but neither completely clears glycogen from type II muscle fibers. *Molecular Therapy* **11**, 48-56 (2005).
2. van der Ploeg, A.T. & Reuser, A.J. Pompe's disease. *The lancet* **372**, 1342-1353 (2008).
3. Ebbink, B.J. et al. Cognitive decline in classic infantile Pompe disease: An underacknowledged challenge. *Neurology* **86**, 1260-1261 (2016).
4. Laforêt, P. et al. Dilative arteriopathy and basilar artery dolichoectasia complicating late-onset Pompe disease. *Neurology* **70**, 2063-2066 (2008).

5. van der Ploeg, A.T. et al. A randomized study of alglucosidase alfa in late-onset Pompe's disease. *N Engl J Med* **362**, 1396-406 (2010).
6. Geel, T., McLaughlin, P., De Leij, L., Ruiters, M. & Niezen-Koning, K. Pompe disease: current state of treatment modalities and animal models. *Molecular genetics and metabolism* **92**, 299-307 (2007).
7. Raben, N. et al. Targeted disruption of the acid alpha-glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen storage disease type II. *J Biol Chem* **273**, 19086-92 (1998).
8. Kohler, L., Puertollano, R. & Raben, N. Pompe Disease: From Basic Science to Therapy. *Neurotherapeutics* **15**, 928-942 (2018).
9. Sidman, R.L. et al. Temporal neuropathologic and behavioral phenotype of 6 neo/6 neo Pompe disease mice. *Journal of Neuropathology & Experimental Neurology* **67**, 803-818 (2008).
10. Fukuda, T. et al. Autophagy and mistargeting of therapeutic enzyme in skeletal muscle in Pompe disease. *Molecular therapy* **14**, 831-839 (2006).
11. Raben, N. et al. Suppression of autophagy in skeletal muscle uncovers the accumulation of ubiquitinated proteins and their potential role in muscle damage in Pompe disease. *Human molecular genetics* **17**, 3897-3908 (2008).
12. Cagin, U. et al. Rescue of advanced Pompe disease in mice with hepatic expression of secretable acid α-glucosidase. *Molecular Therapy* (2020).
13. Turner, S.M. et al. Neuropathology in respiratory-related motoneurons in young Pompe (Gaa−/−) mice. *Respiratory physiology & neurobiology* **227**, 48-55 (2016).
14. Falk, D.J. et al. Peripheral nerve and neuromuscular junction pathology in Pompe disease. *Hum Mol Genet* **24**, 625-36 (2015).
15. Puzzo, F. et al. Rescue of Pompe disease in mice by AAV-mediated liver delivery of secretable acid alpha-glucosidase. *Sci Transl Med* **9** (2017).
16. Falk, D.J. et al. Comparative impact of AAV and enzyme replacement therapy on respiratory and cardiac function in adult Pompe mice. *Mol Ther Methods Clin Dev* **2**, 15007 (2015).
17. Heydemann, A. et al. Latent TGF-β–binding protein 4 modifies muscular dystrophy in mice. *The Journal of clinical investigation* **119**, 3703-3712 (2009).
18. Roberts, N.W., Holley-Cuthrell, J., Gonzalez-Vega, M., Mull, A.J. & Heydemann, A. Biochemical and Functional Comparisons of mdx and Sgcg−/− Muscular Dystrophy Mouse Models. *BioMed research international* **2015** (2015).
19. Flanigan, K.M. et al. LTBP4 genotype predicts age of ambulatory loss in Duchenne muscular dystrophy. *Annals of neurology* **73**, 481-488 (2013).
20. Van Den Bergen, J.C. et al. Validation of genetic modifiers for Duchenne muscular dystrophy: a multicentre study assessing SPP1 and LTBP4 variants. *Journal of Neurology, Neurosurgery & Psychiatry* **86**, 1060-1065 (2015).
21. Weiss, R.B. et al. Long-range genomic regulators of THBS1 and LTBP4 modify disease severity in duchenne muscular dystrophy. *Annals of neurology* **84**, 234-245 (2018).
22. Colella, P. et al. Gene therapy with secreted acid alpha-glucosidase rescues Pompe disease in a novel mouse model with early-onset spinal cord and respiratory defects. *EBioMedicine* **61**, 103052 (2020).
23. Nayak, S. et al. Immune responses and hypercoagulation in ERT for Pompe disease are mutation and rhGAA dose dependent. *PLoS One* **9**, e98336 (2014).
24. Meunier, S. et al. Specificity of the T Cell Response to Protein Biopharmaceuticals. *Front Immunol* **11**, 1550 (2020).
25. Rahemtulla, A. et al. Normal development and function of CD8+ cells but markedly decreased helper cell activity in mice lacking CD4. *Nature* **353**, 180-4 (1991).
26. Fratantoni, J.C., Hall, C.W. & Neufeld, E.F. Hurler and Hunter syndromes: mutual correction of the defect in cultured fibroblasts. *Science* **162**, 570-2 (1968).
27. Dahms, N.M., Lobel, P. & Kornfeld, S. Mannose 6-phosphate receptors and lysosomal enzyme targeting. *J Biol Chem* **264**, 12115-8 (1989).
28. Neufeld, E.F. From serendipity to therapy. *Annu Rev Biochem* **80**, 1-15 (2011).
29. Amalfitano, A. et al. Recombinant human acid alpha-glucosidase enzyme therapy for infantile glycogen storage disease type II: results of a phase I/II clinical trial. *Genet Med* **3**, 132-8 (2001).
30. Van den Hout, H. et al. Recombinant human alpha-glucosidase from rabbit milk in Pompe patients. *Lancet* **356**, 397-8 (2000).
31. Van den Hout, J.M. et al. Long-term intravenous treatment of Pompe disease with recombinant human alpha-glucosidase from milk. *Pediatrics* **113**, e448-57 (2004).
32. Kishnani, P.S. et al. Recombinant human acid [alpha]-glucosidase: major clinical benefits in infantile-onset Pompe disease. *Neurology* **68**, 99-109 (2007).
33. Kishnani, P.S. et al. Cross-reactive immunologic material status affects treatment outcomes in Pompe disease infants. *Mol Genet Metab* **99**, 26-33 (2010).

34. Hahn, S.H. et al. Efficacy, safety profile, and immunogenicity of alglucosidase alfa produced at the 4,000-liter scale in US children and adolescents with Pompe disease: ADVANCE, a phase IV, open-label, prospective study. *Genetics in Medicine* **20**, 1284-1294 (2018).
35. Chakrapani, A., Vellodi, A., Robinson, P., Jones, S. & Wraith, J. Treatment of infantile Pompe disease with alglucosidase alpha: the UK experience. *Journal of inherited metabolic disease* **33**, 747-750 (2010).
36. van Gelder, C.M. et al. Enzyme therapy and immune response in relation to CRIM status: the Dutch experience in classic infantile Pompe disease. *J Inherit Metab Dis* **38**, 305-14 (2015).
37. Schoser, B. et al. Survival and long-term outcomes in late-onset Pompe disease following alglucosidase alfa treatment: a systematic review and meta-analysis. *Journal of neurology* **264**, 621-630 (2017).
38. Orlikowski, D. et al. Recombinant human acid alpha-glucosidase (rhGAA) in adult patients with severe respiratory failure due to Pompe disease. *Neuromuscular Disorders* **21**, 477-482 (2011).
39. Papadopoulos, C. et al. Effect of enzyme replacement therapy with alglucosidase alfa (Myozyme®) in 12 patients with advanced late-onset Pompe disease. *Molecular genetics and metabolism* **122**, 80-85 (2017).
40. Banugaria, S.G. et al. The impact of antibodies on clinical outcomes in diseases treated with therapeutic protein: lessons learned from infantile Pompe disease. *Genet Med* **13**, 729-36 (2011).
41. Ebbink, B.J. et al. Classic infantile Pompe patients approaching adulthood: a cohort study on consequences for the brain. *Dev Med Child Neurol* **60**, 579-586 (2018).
42. Xu, S. et al. Improved efficacy of a next-generation ERT in murine Pompe disease. *JCI Insight* **4** (2019).
43. Parenti, G. et al. A chaperone enhances blood alpha-glucosidase activity in Pompe disease patients treated with enzyme replacement therapy. *Mol Ther* **22**, 2004-12 (2014).
44. Kishnani, P. et al. Duvoglustat HCl increases systemic and tissue exposure of active acid α-glucosidase in Pompe patients co-administered with alglucosidase α. *Molecular Therapy* **25**, 1199-1208 (2017).
45. Zhu, Y. et al. Glycoengineered acid alpha-glucosidase with improved efficacy at correcting the metabolic aberrations and motor function deficits in a mouse model of Pompe disease. *Mol Ther* **17**, 954-63 (2009).
46. Basile, I. et al. Efficient therapy for refractory Pompe disease by mannose 6-phosphate analogue grafting on acid alpha-glucosidase. *J Control Release* **269**, 15-23 (2018).
47. Kang, J.Y. et al. Lysosomal Targeting Enhancement by Conjugation of Glycopeptides Containing Mannose-6-phosphate Glycans Derived from Glyco-engineered Yeast. *Sci Rep* **8**, 8730 (2018).
48. Baik, A. et al. Next-generation antibody-guided enzyme replacement therapy in Pompe disease mice. *Molecular Genetics and Metabolism* **123**, S21 (2018).
49. Maga, J.A. et al. Glycosylation-independent lysosomal targeting of acid α-glucosidase enhances muscle glycogen clearance in pompe mice. *Journal of Biological Chemistry* **288**, 1428-1438 (2013).
50. Yi, H. et al. Antibody-mediated enzyme replacement therapy targeting both lysosomal and cytoplasmic glycogen in Pompe disease. *Journal of Molecular Medicine* **95**, 513-521 (2017).
51. Peng, J. et al. Reveglucosidase alfa (BMN 701), an IGF2-Tagged rhAcid α-Glucosidase, Improves Respiratory Functional Parameters in a Murine Model of Pompe Disease. *Journal of Pharmacology and Experimental Therapeutics* **360**, 313-323 (2017).
52. Byrne, B.J. et al. A study on the safety and efficacy of reveglucosidase alfa in patients with late-onset Pompe disease. *Orphanet journal of rare diseases* **12**, 144 (2017).
53. Koeberl, D.D. et al. Enhanced efficacy of enzyme replacement therapy in Pompe disease through mannose-6-phosphate receptor expression in skeletal muscle. *Mol Genet Metab* **103**, 107-12 (2011).
54. Koeberl, D.D. et al. beta2 Agonists enhance the efficacy of simultaneous enzyme replacement therapy in murine Pompe disease. *Mol Genet Metab* **105**, 221-7 (2012).
55. Li, S. et al. Adjunctive beta2-agonists reverse neuromuscular involvement in murine Pompe disease. *FASEB J* **27**, 34-44 (2013).
56. Koeberl, D.D. et al. Adjunctive albuterol enhances the response to enzyme replacement therapy in late-onset Pompe disease. *FASEB J* **28**, 2171-6 (2014).
57. Koeberl, D.D. et al. Correction of biochemical abnormalities and improved muscle function in a phase I/II clinical trial of clenbuterol in Pompe disease. *Molecular Therapy* **26**, 2304-2314 (2018).
58. Fink, A.L. Chaperone-mediated protein folding. *Physiological reviews* **79**, 425-449 (1999).
59. Parenti, G., Moracci, M., Fecarotta, S. & Andria, G. Pharmacological chaperone therapy for lysosomal storage diseases. *Future Med Chem* **6**, 1031-45 (2014).
60. Flanagan, J.J. et al. The pharmacological chaperone 1-deoxynojirimycin increases the activity and lysosomal trafficking of multiple mutant forms of acid alpha-glucosidase. *Hum Mutat* **30**, 1683-92 (2009).

61. Khanna, R. et al. The pharmacological chaperone AT2220 increases the specific activity and lysosomal delivery of mutant acid alpha-glucosidase, and promotes glycogen reduction in a transgenic mouse model of Pompe disease. *PLoS One* **9**, e102092 (2014).
62. Porto, C. et al. The pharmacological chaperone N-butyldeoxynojirimycin enhances enzyme replacement therapy in Pompe disease fibroblasts. *Mol Ther* **17**, 964-71 (2009).
63. Tsujino, S. et al. Adenovirus-mediated transfer of human acid maltase gene reduces glycogen accumulation in skeletal muscle of Japanese quail with acid maltase deficiency. *Human gene therapy* **9**, 1609-1616 (1998).
64. Zaretsky, J.Z. et al. Retroviral transfer of acid alpha-glucosidase cDNA to enzyme-deficient myoblasts results in phenotypic spread of the genotypic correction by both secretion and fusion. *Hum Gene Ther* **8**, 1555-63 (1997).
65. Pauly, D.F. et al. Complete correction of acid alpha-glucosidase deficiency in Pompe disease fibroblasts in vitro, and lysosomally targeted expression in neonatal rat cardiac and skeletal muscle. *Gene Ther* **5**, 473-80 (1998).
66. Amalfitano, A. et al. Systemic correction of the muscle disorder glycogen storage disease type II after hepatic targeting of a modified adenovirus vector encoding human acid-alpha-glucosidase. *Proc Natl Acad Sci U S A* **96**, 8861-6 (1999).
67. Kyosen, S.O. et al. Neonatal gene transfer using lentiviral vector for murine Pompe disease: long-term expression and glycogen reduction. *Gene Ther* **17**, 521-30 (2010).
68. Doerfler, P.A. et al. Targeted approaches to induce immune tolerance for Pompe disease therapy. *Mol Ther Methods Clin Dev* **3**, 15053 (2016).
69. Sun, B. et al. Correction of multiple striated muscles in murine Pompe disease through adeno-associated virus-mediated gene therapy. *Mol Ther* **16**, 1366-71 (2008).
70. Han, S.O. et al. Low-Dose Liver-Targeted Gene Therapy for Pompe Disease Enhances Therapeutic Efficacy of ERT via Immune Tolerance Induction. *Mol Ther Methods Clin Dev* **4**, 126-136 (2017).
71. Colella, P. et al. AAV Gene Transfer with Tandem Promoter Design Prevents Anti-transgene Immunity and Provides Persistent Efficacy in Neonate Pompe Mice. *Mol Ther Methods Clin Dev* **12**, 85-101 (2019).
72. Fraites, T.J., Jr. et al. Correction of the enzymatic and functional deficits in a model of Pompe disease using adeno-associated virus vectors. *Mol Ther* **5**, 571-8 (2002).
73. Schuster, D.J. et al. Biodistribution of adeno-associated virus serotype 9 (AAV9) vector after intrathecal and intravenous delivery in mouse. *Frontiers in neuroanatomy* **8**, 42 (2014).
74. van Til, N.P. et al. Lentiviral gene therapy of murine hematopoietic stem cells ameliorates the Pompe disease phenotype. *Blood, The Journal of the American Society of Hematology* **115**, 5329-5337 (2010).
75. Sun, B. et al. Correction of glycogen storage disease type II by an adeno-associated virus vector containing a muscle-specific promoter. *Mol Ther* **11**, 889-98 (2005).
76. Sun, B. et al. Efficacy of an adeno-associated virus 8-pseudotyped vector in glycogen storage disease type II. *Mol Ther* **11**, 57-65 (2005).
77. Franco, L.M. et al. Evasion of immune responses to introduced human acid alpha-glucosidase by liver-restricted expression in glycogen storage disease type II. *Mol Ther* **12**, 876-84 (2005).
78. Mah, C. et al. Physiological correction of Pompe disease by systemic delivery of adeno-associated virus serotype 1 vectors. *Mol Ther* **15**, 501-7 (2007).
79. Koeberl, D.D. & Kishnani, P.S. Immunomodulatory gene therapy in lysosomal storage disorders. *Curr Gene Ther* **9**, 503-10 (2009).
80. Mah, C. et al. Sustained correction of glycogen storage disease type II using adeno-associated virus serotype 1 vectors. *Gene Ther* **12**, 1405-9 (2005).
81. Mah, C.S. et al. Gel-mediated delivery of AAV1 vectors corrects ventilatory function in Pompe mice with established disease. *Mol Ther* **18**, 502-10 (2010).
82. Rucker, M. et al. Rescue of enzyme deficiency in embryonic diaphragm in a mouse model of metabolic myopathy: Pompe disease. *Development* **131**, 3007-19 (2004).
83. Elmallah, M.K. et al. Sustained correction of motoneuron histopathology following intramuscular delivery of AAV in pompe mice. *Mol Ther* **22**, 702-12 (2014).
84. Qiu, K., Falk, D.J., Reier, P.J., Byrne, B.J. & Fuller, D.D. Spinal delivery of AAV vector restores enzyme activity and increases ventilation in Pompe mice. *Mol Ther* **20**, 21-7 (2012).
85. Cresawn, K.O. et al. Impact of humoral immune response on distribution and efficacy of recombinant adeno-associated virus-derived acid alpha-glucosidase in a model of glycogen storage disease type II. *Hum Gene Ther* **16**, 68-80 (2005).
86. Ziegler, R.J. et al. Ability of adeno-associated virus serotype 8-mediated hepatic expression of acid alpha-glucosidase to correct the biochemical and motor function deficits of presymptomatic and symptomatic Pompe mice. *Hum Gene Ther* **19**, 609-21 (2008).

87. Wang, G. et al. Assessment of toxicity and biodistribution of recombinant AAV8 vector-mediated immunomodulatory gene therapy in mice with Pompe disease. *Mol Ther Methods Clin Dev* **1**, 14018 (2014).
88. Todd, A.G. et al. Correcting Neuromuscular Deficits With Gene Therapy in Pompe Disease. *Ann Neurol* **78**, 222-34 (2015).
89. Falk, D.J. et al. Intrapleural administration of AAV9 improves neural and cardiorespiratory function in Pompe disease. *Mol Ther* **21**, 1661-7 (2013).
90. ElMallah, M.K. et al. Retrograde gene delivery to hypoglossal motoneurons using adeno-associated virus serotype 9. *Hum Gene Ther Methods* **23**, 148-56 (2012).
91. Corti, M. et al. Safety of Intradiaphragmatic Delivery of Adeno-Associated Virus-Mediated Alpha-Glucosidase (rAAV1-CMV-hGAA) Gene Therapy in Children Affected by Pompe Disease. *Hum Gene Ther Clin Dev* **28**, 208-218 (2017).
92. Smith, B.K. et al. Phase I/II trial of adeno-associated virus-mediated alpha-glucosidase gene therapy to the diaphragm for chronic respiratory failure in Pompe disease: initial safety and ventilatory outcomes. *Hum Gene Ther* **24**, 630-40 (2013).
93. Byrne, P.I. et al. Phase I/II trial of diaphragm delivery of recombinant adeno-associated virus acid alpha-glucosidase (rAAaV1-CMV-GAA) gene vector in patients with Pompe disease. *Hum Gene Ther Clin Dev* **25**, 134-63 (2014).
94. Smith, B.K. et al. Inspiratory muscle conditioning exercise and diaphragm gene therapy in Pompe disease: Clinical evidence of respiratory plasticity. *Exp Neurol* **287**, 216-224 (2017).
95. Calcedo, R. et al. Class I-restricted T-cell responses to a polymorphic peptide in a gene therapy clinical trial for α-1-antitrypsin deficiency. *Proceedings of the National Academy of Sciences* **114**, 1655-1659 (2017).
96. Mendell, J.R. et al. Dystrophin immunity in Duchenne's muscular dystrophy. *New England Journal of Medicine* **363**, 1429-1437 (2010).
97. Mendell, J.R. et al. Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. *N Engl J Med* **377**, 1713-1722 (2017).
98. Childers, M.K. et al. Gene therapy prolongs survival and restores function in murine and canine models of myotubular myopathy. *Science translational medicine* **6**, 220ra10-220ra10 (2014).
99. Le Guiner, C. et al. Long-term microdystrophin gene therapy is effective in a canine model of Duchenne muscular dystrophy. *Nature communications* **8**, 1-15 (2017).
100. Mack, D.L. et al. Systemic AAV8-mediated gene therapy drives whole-body correction of myotubular myopathy in dogs. *Molecular Therapy* **25**, 839-854 (2017).
101. Audentes. (https://www.joshuafrase.org/get-involved/recensus-study.php, 2020).
102. Sun, B. et al. Antibody formation and mannose-6-phosphate receptor expression impact the efficacy of muscle-specific transgene expression in murine Pompe disease. *The journal of gene medicine* **12**, 881-891 (2010).
103. Zhang, P. et al. Immunodominant liver-specific expression suppresses transgene-directed immune responses in murine pompe disease. *Hum Gene Ther* **23**, 460-72 (2012).
104. Sun, B. et al. Immunomodulatory gene therapy prevents antibody formation and lethal hypersensitivity reactions in murine pompe disease. *Mol Ther* **18**, 353-60 (2010).
105. Bond, J.E., Kishnani, P.S. & Koeberl, D.D. Immunomodulatory, liver depot gene therapy for Pompe disease. *Cell Immunol* (2017).
106. Sun, B. et al. Enhanced efficacy of an AAV vector encoding chimeric, highly secreted acid α-glucosidase in glycogen storage disease type II. *Molecular Therapy* **14**, 822-830 (2006).
107. Petersen, T.N., Brunak, S., Von Heijne, G. & Nielsen, H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature methods* **8**, 785-786 (2011).
108. Perrin, G.Q. et al. Dynamics of antigen presentation to transgene product-specific CD4(+) T cells and of Treg induction upon hepatic AAV gene transfer. *Mol Ther Methods Clin Dev* **3**, 16083 (2016).
109. Bortolussi, G. et al. Life-long correction of hyperbilirubinemia with a neonatal liver-specific AAV-mediated gene transfer in a lethal mouse model of Crigler-Najjar Syndrome. *Hum Gene Ther* **25**, 844-55 (2014).
110. Meliani, A. et al. Antigen-selective modulation of AAV immunogenicity with tolerogenic rapamycin nanoparticles enables successful vector re-administration. *Nat Commun* **9**, 4098 (2018).
111. Kuranda, K. et al. Exposure to wild-type AAV drives distinct capsid immunity profiles in humans. *J Clin Invest* **128**, 5267-5279 (2018).
112. Corti, M. et al. Evaluation of readministration of a recombinant adeno-associated virus vector expressing acid alpha-glucosidase in Pompe disease: preclinical to clinical planning. *Human Gene Therapy Clinical Development* **26**, 185-193 (2015).
113. Lim, J.-A. et al. Intravenous Injection of an AAV-PHP. B vector encoding human acid α-glucosidase rescues both muscle and CNS defects in murine Pompe disease. *Molecular Therapy-Methods & Clinical Development* **12**, 233-245 (2019).

114. Lee, N.C. et al. A Neuron-Specific Gene Therapy Relieves Motor Deficits in Pompe Disease Mice. *Mol Neurobiol* **55**, 5299-5309 (2018).
115. Hordeaux, J. et al. Long-term neurologic and cardiac correction by intrathecal gene therapy in Pompe disease. *Acta Neuropathol Commun* **5**, 66 (2017).
116. Spampanato, C. et al. Transcription factor EB (TFEB) is a new therapeutic target for Pompe disease. *EMBO Mol Med* **5**, 691-706 (2013).
117. Gatto, F. et al. AAV-mediated transcription factor EB (TFEB) gene delivery ameliorates muscle pathology and function in the murine model of Pompe Disease. *Sci Rep* **7**, 15089 (2017).
118. Clayton, N.P. et al. Antisense Oligonucleotide-mediated Suppression of Muscle Glycogen Synthase 1 Synthesis as an Approach for Substrate Reduction Therapy of Pompe Disease. *Mol Ther Nucleic Acids* **3**, e206 (2014).
119. Douillard-Guilloux, G. et al. Restoration of muscle functionality by genetic suppression of glycogen synthesis in a murine model of Pompe disease. *Hum Mol Genet* **19**, 684-96 (2010).
120. Douillard-Guilloux, G. et al. Modulation of glycogen synthesis by RNA interference: towards a new therapeutic approach for glycogenosis type II. *Hum Mol Genet* **17**, 3876-86 (2008).
121. Sukigara, S. et al. Muscle glycogen storage disease 0 presenting recurrent syncope with weakness and myalgia. *Neuromuscular Disorders* **22**, 162-165 (2012).
122. van der Wal, E., Bergsma, A.J., Pijnenburg, J.M., van der Ploeg, A.T. & Pijnappel, W.P. Antisense oligonucleotides promote exon inclusion and correct the common c.-32-13T> G GAA splicing variant in Pompe disease. *Molecular Therapy-Nucleic Acids* **7**, 90-100 (2017).
123. Goyenvalle, A., Leumann, C. & Garcia, L. Therapeutic potential of tricyclo-DNA antisense oligonucleotides. *Journal of neuromuscular diseases* **3**, 157-167 (2016).
124. Mingozzi, F. & High, K.A. Overcoming the host immune response to adeno-associated virus gene delivery vectors: the race between clearance, tolerance, neutralization, and escape. *Annual review of virology* **4**, 511-534 (2017).
125. Colella, P., Ronzitti, G. & Mingozzi, F. Emerging issues in AAV-mediated in vivo gene therapy. *Molecular Therapy-Methods & Clinical Development* **8**, 87-104 (2018).

Chapter 3

Glycogen synthase 1 inhibition as substrate reduction therapy for Pompe disease

Unpublished data

1. Introduction

Pompe disease (PD) is the first and probably the more comprehensively characterized lysosomal storage disorder (LSD) that manifests as a metabolic myopathy. In addition, animal models mimicking the human disease have been created facilitating the translational research works.[1, 2] For these reasons, over the course of the last few decades, several therapeutic approaches aimed at addressing the pathology were explored, already rviewed in the previous chapters.

Substrate reduction therapy (SRT) is one of theses therapeutic modality.[3-5] This strategy relies on the modulation of glycogen synthesis by reduction of the activity of the muscular glycogen synthase enzyme (GYS1) to reduce the accumulation of glycogen. We proposed in this study new SRT approaches with the use of artificial miRNA-based gene silencing and CRISPR/Cas9-mediated genome editing.

In these works, we developed miRNA sequences targeting specifically *Gys1* mRNA (miR-*Gys1*) and explored their efficacy to reduce glycogen synthesis in murine myoblasts and in GSDII mouse models. An efficient *in vitro* and *in vivo* inhibition of GYS1 was obtained. Furthermore, efficient muscle transduction with AAV9 vectors expressing miR-*Gys1* in newborn GSDII mice resulted in strong inhibition of GYS1 expression and in decrease of glycogen accumulation. In addition, a complete normalization of glycogen content was achieved in hearts of GSD II mice treated as neonates with CRISPR/Cas9-mediated genome editing. Further development are needed, notably in larger animal cohorts, to confirm these results and make the approach safer and more effective in skeletal muscles. These SRT strategies could constitute an efficient approach for preventing glycogen build up in PD, and may complement or be used as an alternative to ERT and gene replacement therapy.

2. Materials and methods

2.1 Test Items

miR-*Gys1* and *Gys1*-gRNA design and cloning

Benchling software (www.benchling.com) was used to design the gRNA targeting the exonic sequences of mouse *Gys1* gene. The target sequence consisted of 21-nt followed by a PAM sequence which is require for *Staphylococcus aureus* Cas9 (saCas9) to generate a double stranded break. Different gRNA were selected based on their predicted on-target an off-target scores and were cloned in a pX-601 backbone, downstream a polymerase III (Pol III) promoter, using *KpnI*/*NotI* restrictions enzymes. Then, saCas9 was added in the expression cassette and placed under the control of a muscular specific promoter or downstream an ubiquitous promoter. Cloning was performed using XhoI/AgeI restrictions enzymes, following manufacturer's instructions.

BLOCK-iT™ RNAi designer software (https://rnaidesigner.thermofisher.com/rnaiexpress/) was used to design artificial miRNA sequences targeting *Gys1* mRNA (NM 030678.3). Each selected *Gys1* mRNA-specific miRNA sequence (miR-*Gys1*) was annealed into double stranded oligonucleotides and cloned downstream a strong polymerase II (Pol II) promoter, using The BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Thermo Fischer Scientific, Waltham, MA), following manufacturer's instructions. *NcoI*/*XhoI* fragment containing eGFP and miR-*Gys1* sequences was introduced in a pAAV backbone, comprising

the wild-type AAV2 ITRs, a Pol II promoter, the woodchuck hepatitis post-transcriptional regulatory element (WPRE) and a polyA signal.

Production of AAV vectors

AAV vectors used in this study were produced using a slight modification of the adenovirus-free transient transfection methods described.[6] Briefly, adherent HEK293 cells grown in roller bottles were transfected with the three plasmids containing the adenovirus helper proteins, the AAV Rep and Cap genes, and the ITR-flanked transgene expression cassette. 72 hours after transfection, cells were harvested, lysed by sonication, and treated with Benzonase® (Merck-Millipore, Darmstadt, Germany). Vectors were then purified using two successive ultracentrifugation rounds in cesium chloride density gradients. Full capsids were collected, the final product was formulated in sterile phosphate buffered saline containing 0.001% of Pluronic (Sigma Aldrich, Saint Louis, MO), and stored at -80 °C.

Titers of AAV vector stocks were determined using SDS-PAGE, followed by SYPRO Ruby protein gel stain (Invitrogen, Carlsbad, CA) and band quantification using ImageJ software. All vector preparations used in the studies were quantified side by side at least 3 times before use.

2.2 GYS1 expression in C2C12 cell line

C2C12 immortalized mouse myoblast cells were cultured in DMEM-FBS10% medium at 37ºC with 5% CO_2.

Six-well culture plates containing 80% confluent C2C12 cells in opti-MEM medium (Thermo Fisher Scientific, Waltham, MA) were transfected with pAAV–eGFP–miR-*Gys1* plasmids using Lipofectamine™ 3000 (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. pAAV–eGFP transfected cells were included as control. 72hrs after transfection, GFP expression was observed under fluorescent microscope (EVOS FLoid Cell Imaging Station, Thermo Fisher Scientific, Waltham, MA) and then cells were harvested and pellets were frozen at -20°C until processing.

Pelleted cells were resuspended in lysis buffer (0.4M Tris-phosphate, pH7.8; 40% glycerol; 2mM EDTA; 0.4M $MgCl_2$; 0.04% Triton X-100; 0.4M DTT) supplemented with protease inhibitor cocktail (cOmplete™, Roche Diagnosis, Basel, Switzerland). Lysates were centrifuged at 12.000g for 5min and the protein-containing supernatant was recovered. Total proteins concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) following manufacturer instructions and protein extracts were separated on a 4-12% bis-tris polyacrylamide gel (Thermo Fisher Scientific, Waltham, MA). The same amount of protein was loaded in each lane. The gel was transferred onto a nitrocellulose membrane and blotted with an anti-GYS1 antibody (Thermo Fisher Scientific, Waltham, MA) and an anti-GAPDH antibody (Thermo Fisher Scientific, Waltham, MA) used as loading control. Secondary antibodies and detection system were from Li-Cor Biosciences (Lincoln, NE).

2.3 *In vivo* studies

Mice

Mouse studies were performed according to the French and European legislation on animal care and experimentation (2010/63/EU) and approved by the local Institutional Ethical Board (protocols no. 2016-019B, apafis no. 9265 and 2017-011B, apafis no. 13643). Mice were kept in a temperature-controlled environment with 12/12-hour light–dark cycle. They received a standard chow diet and water ad libitum.

The *Gaa*$^{-/-}$ mice were purchased from the Jackson Laboratory (B6;129-Gaa^{tm1Rabn}/J, stock no. 004154, 6neo). This mouse model was originally generated Raben et al.[1] *Gaa*$^{+/+}$ mice are derived from the same colony and breed separately. CD4-deficient Pompe disease mice were generated by successive crossings of *Gaa*$^{-/-}$ and *Cd4*$^{-/-}$ mice. Genotypes were confirmed by polymerase chain reaction (PCR) performed on genomic DNA with oligonucleotides specific for the mutated regions in each gene.

Gaa (mutant)	forward 5'-CGTTGGCTACCCGTGATATT-3'
Gaa (wild-type)	forward 5'-TCCTGAGCCCAAACACTTCT-3'
Gaa	reverse 5'-ATTGTTGCACAACGCTCTTG-3'
Cd4 (mutant)	forward 5'-GTGTTGGGTCGTTTGTTCG-3'
Cd4 (wild-type)	forward 5'-CCTCTTGGTTAATGGGGGAT-3'
Cd4	reverse 5'-TTTTTCTGGTCCAGGGTCAC-3'

Gene transfer procedures

Intramuscular treatment of adult mice

Three-four months old *Cd4*$^{-/-}$*Gaa*$^{-/-}$ mice, were injected in the left tibialis anterior with 1x10^{11}vg of AAV9 vectors expressing miR-*Gys1* or unspecific-targeting miRNA (n = 3/4 per treatment group). Injected and contralateral muscles were collected at sacrifice, 2 weeks post-treatment and were frozen in liquid nitrogen.

Three-four months old *Gaa*$^{-/-}$ mice, were injected in the left gastrocnemius with 6x10^{11}vg of AAV9 vectors expressing CRISPR/Cas9 and *Gys1*-specific gRNA (*Gys1*-gRNA) or unspecific gRNA (n = 5 per treatment group). Injected and contralateral muscles were collected at sacrifice, 2 months post-treatment and were frozen in liquid nitrogen.

Intravenous treatment of neonate mice

Gaa$^{-/-}$ neonates were injected in the temporal vein with 1.5x10^{11}vg of AAV9 vectors expressing miR-*Gys1* or CRISPR/Cas9 and *Gys1*-gRNA (n = 3/4 per treatment group). Mice were followed during 6months. Untreated age-matched *Gaa*$^{-/-}$ littermates and *Gaa*$^{+/+}$ mice were used as control. At the time of sacrifice, some organs and muscles were collected and were frozen in liquid nitrogen.

Vector genome copy number analysis

Snap-frozen tissues were homogenized in PBS with the use of FastPrep lysis tubes (MP Biomedicals, Ohio, USA), followed by centrifugation 20min at 10,000×g to collect the supernatant. Vector DNA was extracted from tissue homogenates using KingFisher™ automated system (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions and vector genome copy number (VGCN) was quantified by qPCR as previously described,[7] using specific primers for the GFP:

GFP	forward 5'- AGTCCGCCCTGAGCAAAGA-3'
	reverse 5'- GCGGTCACGAACTCCAGC-3'
TITIN	forward 5'-AGAGGTAGTATTGAAAACGAGCGG-3'
	reverse 5'-GCTAGCGCTCCCGCTGCTGAAGCTG-3'

InDels determination

Genomic DNA from muscle homogenate was isolated using DNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and a PCR was realized to amplify the region surrounding the expected break site. After purification of PCR fragments, using Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions, they were sent for sequencing (Brooks Life Science, Manchester; UK). The resulting sequence trace files (.ab1 format) were then uploaded into the TIDE web tool (http://tide.nki.nl) for quality control and InDels determination as previously described.[8]

Western blot analysis

Total proteins concentration of tissue homogenates, prepared as described above, was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) following manufacturer instructions and protein extracts were separated on a 4-12% bis-tris polyacrylamide gel (Thermo Fisher Scientific, Waltham, MA). The same amount of protein was loaded in each lane. The gel was transferred onto a nitrocellulose membrane and blotted with anti-GYS1 and/or anti-GYS2 antibodies (Thermo Fisher Scientific, Waltham, MA). Anti-GAPDH or anti-Tubulin antibodies (Thermo Fisher Scientific, Waltham, MA) were used as loading control. Secondary antibodies and detection system were from Li-Cor Biosciences (Lincoln, NE).

Glycogen content measurement

Glycogen content was analyzed in tissue homogenates, prepared as previously described. 20µl of homogenate properly diluted was mixed with 55µl of distilled water, incubated for 5min at 95°C, and cooled at 4°C. Next, glycogen contained in samples was digested with amyloglucosidase from Aspergillus Niger (Sigma Aldrich, Saint-Louis, MO), in order to release the glucose monomers. For that, 25µl of amyloglucosidase diluted 1:50 in 0.1M potassium acetate pH5.5 was added to each sample. A control reaction without amyloglucosidase was done in parallel for each sample. Both digestion and control samples were incubated at 37°C for 90min, followed by 5min at 95°C. Glycogen content was determined indirectly by quantifying the number of glucose molecules released per gram of protein, with the use of a colorimetric glucose assay kit (Sigma Aldrich, Saint-Louis, MO).

Glucose assay was performed according to the manufacturer's instructions. Absorbance was measured with an EnSpire alpha plate reader (PerkinElmer) at 540 nm.

Statistical analysis

Results are expressed as mean ± SD as described in the text. The Prism package (Graph Pad Software, La Jolla, CA) was used to analyze data. Statistical analysis has been performed by one way ANOVA or unpaired t-test as described in the text. Values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1 Construction of vectors expressing miRNA targeted to the murine *Gys1* mRNA

Located on chromosome 7, the mouse glycogen synthase gene (*Gys1)*, which encodes the rate-limiting enzyme for glycogen synthesis of skeletal muscle, was chosen as candidate gene for SRT strategy for Pompe disease. Composed by 16 exons, mouse *Gys1* is transcribed in a 3681bp mRNA (**Figure 3.1A**), on which *in silico* analyses were performed for the design of specific synthetic miRNA sequences. Out of more than 300 predicted candidates, 7 miRNAs were selected targeting not only the 3' untranslated region (UTR), that naturally presents regulatory domains that allow endogenous miRNA binding,[9] but targeting the different regions of the *Gys1* mRNA sequence.

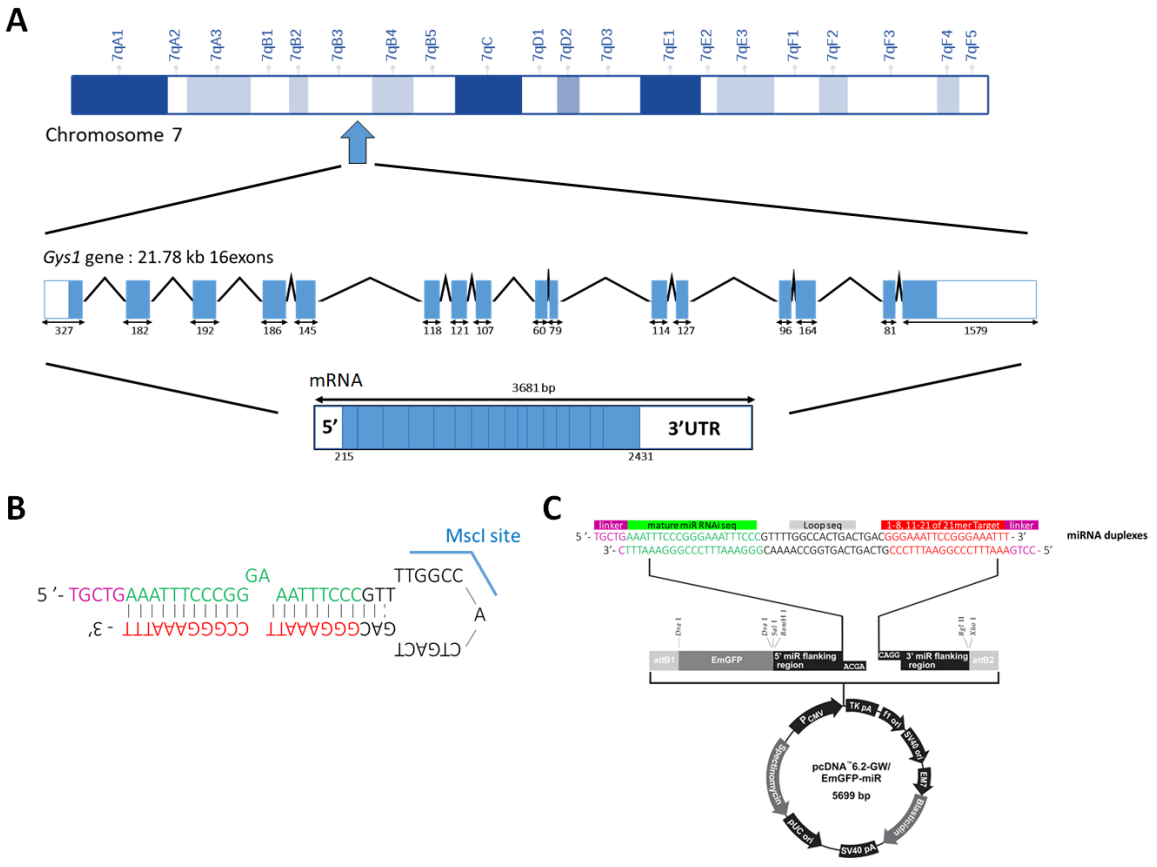

Figure 3.1 miRNA vectors design and cloning
(A) Schematic representation of the mouse Gys1 gene structure, with its chromosomal localization, exons count, size and its mRNA transcript.(B-C) Representative illustration of the pre-miRNA structure and its directional cloning into the shuttle vector. (B) the pre-miRNA is composed by a 5'overhang required for directional cloning (in purple), a 21-nt antisense

sequence representing the mature miRNA derived from the target gene (in green), followed by a 19-nt spacer to form the terminal loop (in black), including the restriction site MscI and a sense target sequence with 2 nucleotides removed creating an internal loop (in red). (C) After annealing of complementary oligonucleotides, miRNA duplexes were cloned into a shuttle plasmid containing mir-155 flanking regions allowing correct miRNA processing (pcDNA6.2 GW-EmGFP-miR).

In order to facilitate the cloning, artificial miRNAs were synthetized as oligonucleotides. For optimized knockdown results, aside from the mature antisense miRNA sequence (derived from the target gene), miRNA oligonucleotides were designed with 5' and 3' overhangs that allow directional cloning, a short spacer of 19 nucleotides to form the terminal loop and a short sense target sequence with 2 deleted nucleotides to create an internal loop. An Msc I site was incorporated in the terminal loop to aid in sequence analysis (**Figure 3.1B**). After oligonucleotides annealing, double stranded duplexes were cloned into a commercialized expression plasmid containing murine miR-155 flanking sequences (**Figure 3.1C**) that allow proper processing of the miRNAs,[10] and then incorporated into an AAV expression cassette under the control of a strong Pol II promoter flanked by AAV2 ITRs (pAAV–eGFP–miR-*Gys1*) (**Figure 3.2A** and Materials and Methods section). A first *in vitro* miRNAs screening was then performed.

3.2 miRNA-mediated reduction of GYS1 protein levels in murine myoblasts

In order to evaluate the efficacy of the *Gys1* mRNA-specific miRNA sequences (named hereafter miR-*Gys1*) to silence gene expression, C2C12 murine myoblasts were transfected with the different pAAV–eGFP–miR-*Gys1* generated or with pAAV–eGFP as negative control. After 72 hours, efficient gene silencing was observed in miR-*Gys1* transfected C2C12 myoblasts as shown by western-blot analysis of GYS1 protein expression levels (**Figure 3.2B**). In miR-*Gys1* transfected cells, reduction in GYS1 has reached 35% for the most effective miRNA (miR-26) (**Figure 3.2B**).

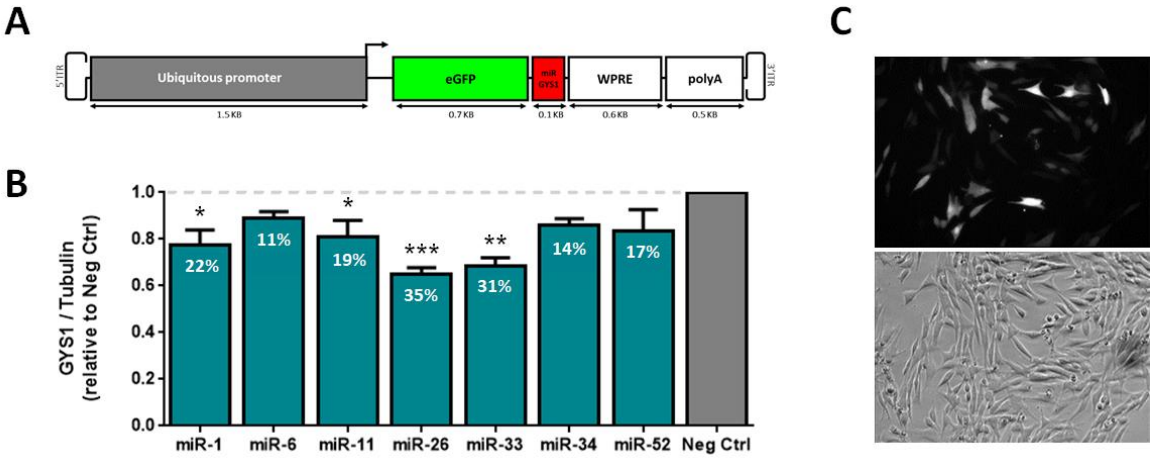

Figure 3.2 In vitro evaluation of miR-Gys1
*(A) AAV-eGFP-miR-Gys1 representative expression cassette. The cassette is flanked by the AAV2 inverted repeated terminal (ITR) sequences and contains the cDNA sequences coding for GFP (eGFP) and miR-Gys1 placed under the control of a strong ubiquitous Pol II promoter, the woodchuck hepatitis post-transcriptional regulatory element (WPRE) and a polyadeneylation signal (polyA). (B) In vitro analysis of mir-Gys1 efficacy. C2C12 cells were cultured in 6-well plates and then immediately transfected with the miR-Gys1-expressing plasmids indicated in the histogram, using Lipofectamine 3000 in opti-MEM medium. 72 h post-transfection, the cells were collected, lysed and analyzed by Western blot. The normalized values of Gys1expression are expressed as a change factor relative to pAAV–eGFP-transfected cells (Neg Ctrl). Percentage of reduction of Gys1 expression are indicated. Each condition was tested in duplicate and the results are represented by the mean ± standard deviation. Statistical analysis were performed by one-way ANOVA(.vs Neg Ctrl; * = p<0.05, ** = p<0.01, *** = p<0.001). (C) representative images of C2C12 cells transfected with pAAV-eGFP-miR-Gys1 to fluorescent channel (positive GFP cells) or bright field (total cells).*

The efficacy of miRNA-mediated reduction of GYS1 expression in these myoblasts was to some extent variable and moderate but nevertheless allowed the identification of some interesting candidates (miR-26 and miR-33) (**Figure 3.2B**). These results were particularly encouraging, especially considering that the rate of transfection of this cell type was quite low, despite efforts made to optimize it, and estimated around 30-40% (**Figure 3.2C**). Indeed, western-blot analysis was done on the pool of harvested cells, composed by a majority of non-transfected cells, resulting in an underestimated efficacy of RNA interference.

These results demonstrate miRNA efficacy in silencing *Gys1* gene expression in mouse myoblasts.

3.3 GYS1 knock down does not correct glycogen build up in skeletal muscle of GSD II mice

miR-*Gys1* efficacy was then tested *in vivo* in order to evaluate the impact of GYS1 inhibition on the muscle glycogen content. To do this, AAV9 expressing miR-*Gys1* (**Figure 3.2A**) or unspecific miRNA were produced and directly injected into tibialis anterior muscle of adult GSD II mice ($1x10^{11}$vg/mouse). Two weeks after injection, mice were sacrificed and injected muscles were harvested for GYS1 protein and glycogen storage analysis.

As observed *in vitro* in mouse myoblasts, the three miR-*Gys1* effectively reduced the GYS1 protein expression in the GSD II mouse muscle. The quantification of GYS1 protein levels revealed a near 60% decrease in all miR-*Gys1*-injected muscles as compared with non-specific miRNA injected ones (**Figure 3.3A**). Interestingly, after intramuscular injection, miR-6 appeared as effective as the other miRNAs to interfere with the *Gys1* mRNA, which was not the case *in vitro*.

However, this level of GYS1 inhibition obtained did not appear to be sufficient to correct the accumulated glycogen content in GSD II mice muscles (**Figure 3.3B)**. These results were confirmed by a second approach attempted for *Gys1* silencing based on genome editing, using CRISPR/Cas9 tools and *Gys1*-specific guide RNA. In GSD II mice, despite 35% of indels generated in the *Gys1* gene (**Figure 3.3C**), resulting in 65% reduction of GYS1 protein level (**Figure 3.3D**), no impact on glycogen content was observed in gene editing-treated muscles (**Figure 3.3E**).

The absence of glycogen clearance in skeletal muscle after GYS inhibition may be explained by the inability of adult GSD II mice to degrade the glycogen already accumulated from birth.

These results in lysosomes also support that the miRNA-mediated reduction of GYS1 protein levels is not sufficient to rescue glycogen accumulation in skeletal muscle of GSD II mice. In order to evaluate if the miRNA-mediated SRT strategy could prevent the glycogen accumulation, we further investigated the miR-*Gys1* efficacy in Pompe mice injected right after birth when glyogen accumulation is much lower.[1, 11]

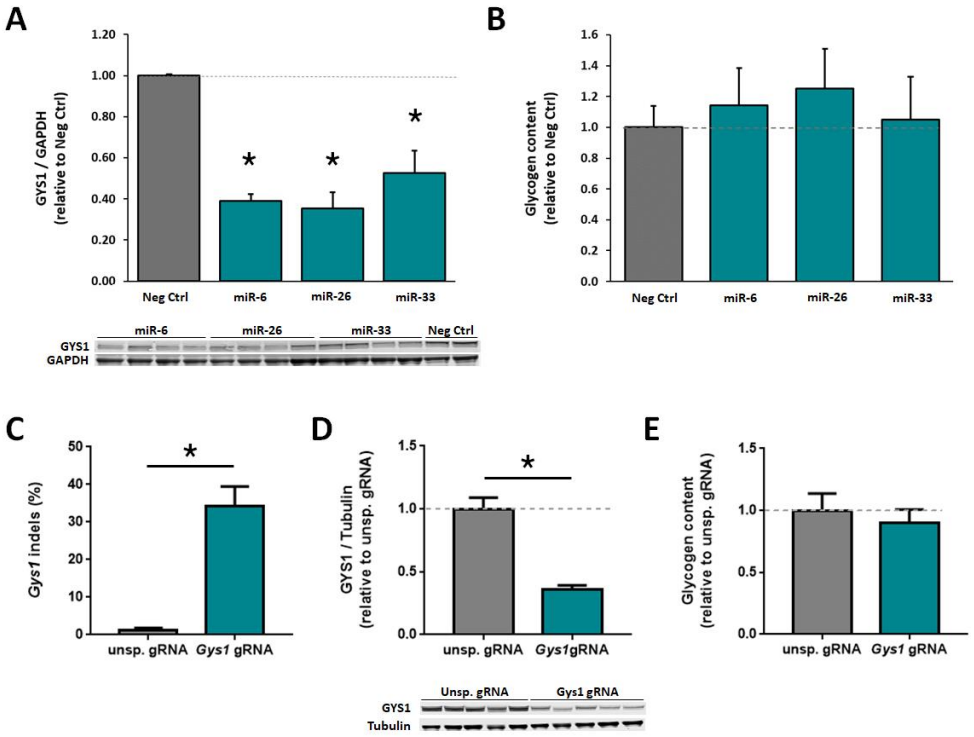

Figure 3.3 GYS1 silencing in adult GSD II mice after intramuscular treatment
(A-B) 3-4 month-old Cd4$^{-/-}$ Gaa$^{-/-}$ mice were treated intramuscularly with 1x10^{11}vg/muscle of AAV vectors expressing miR-Gys1 (miR-6, miR-26, miR-33) or an unspecific miRNA (Neg Ctrl). These mice were sacrificed 2 weeks after injection for biochemical analysis (n=3-4 per group). Data are represented as mean ± sd. Statistical analysis were performed by Anova (= p<0.05 vs Neg Ctrl). (A) GYS1 expression analysis by Western Blot, performed on injected tibialis anterior lysates (50µg of total proteins). Quantification of the bands intensity is plotted on the top. GYS1 expression was normalized by GAPDH expression and expressed relative to the Neg Ctrl values. (B) Determination of the glycogen content present in the tibialis anterior lysates. (C-E) 3-4 month-old Gaa$^{-/-}$ mice were treated intramuscularly with 6x10^{11}vg/muscle of AAV vectors expressing the saCas9 and a Gys1-targeting gRNA (Gys1 gRNA) or an unspecific gRNA (unsp. gRNA). These mice were sacrificed 2 months after injection for biochemical analysis (n=5 per group). (C) Quantification of InDels frequency by sequencing and TIDE analysis. (D) GYS1 expression analysis by Western Blot, performed on injected gastrocnemius lysates (50µg of total proteins). Quantification of the bands intensity is plotted on the top. GYS1 expression was normalized by Tubulin expression and expressed relative to the unsp. gRNA values. (E) Determination of the glycogen content present in the gastrocnemius lysates. Data are represented as mean ± sd. Statistical analysis were performed by unpaired t-test (* = p<0.05).*

3.4 GYS1 inhibition prevents glycogen accumulation in GSD II mice

GSD II mice neonates, presenting low initial glycogen content (data not shown), were intravenously injected with AAV9 expressing miR-*Gys1* (**Figure 3.2A**) or unspecific miRNA at the dose of 1.5x10^{11}vg/mouse. Initially planned with 6 months follow-up, the study was prematurely stopped because of the sudden death of miR-*Gys1*-treated mice (6 out of 10) in the first months following the treatment. The number of mice was then reduced to 1 or 2 per treated group, making not possible any statistical analysis of the results that will follow. The surviving mice and age-matched untreated animals were sacrificed and vector genome copy number (VGCN), GYS1 protein and glycogen storage analysis was performed on different tissues, including heart, quadriceps and liver.

VGCN analysis in tissues of treated animals revealed the persistence of the AAV vectors genome in heart, skeletal muscle and liver, several months post injection (**Figure 3.4A-C**). No difference between mice treated with either AAV expressing miR-6, miR-26 or miR-33 was observed, implying a similar infectivity and biodistribution of the three vectors.

Reduced GYS1 protein expression levels measured in heart and skeletal muscles of treated mice evidenced the miR-*Gys1* efficacy. Actually, a near 70% and 95% silencing was achieved in the heart and the quadriceps respectively, independently of the miR-*Gys1* treatment (**Figure 3.4D-E**). Moreover, the specificity of action of the designed miRNA-*Gys1* was demonstrated by the absence of reduction of GYS2 in the liver of treated mice (**Figure 3.4F**).

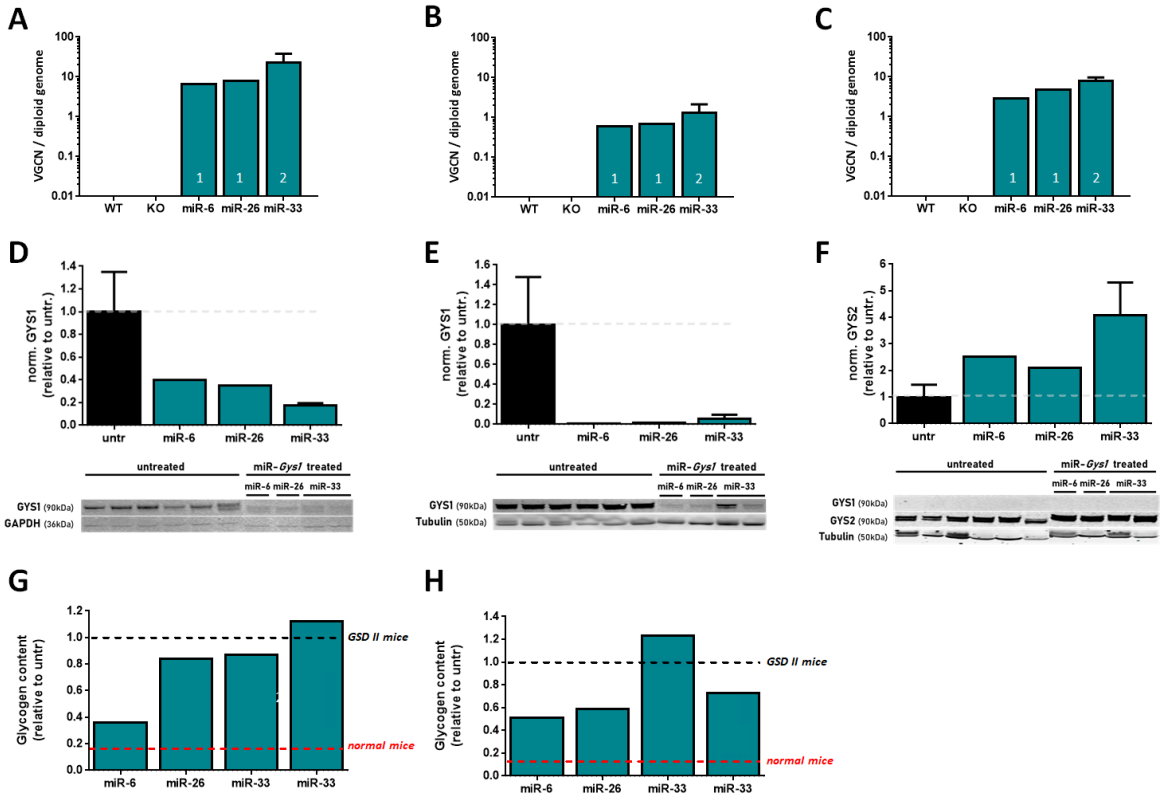

Figure 3.4 miRNA-mediated reduction of GYS1 protein levels partially prevents glycogen accumulation in GSD II mice
Neonates Gaa$^{-/-}$ mice (0-2 days, n=3-4 per group) were treated intravenously with 1.5x10^{11}vg/mouse of AAV vectors expressing miR-Gys1 (miR-6, miR-26, miR-33). 6 out of 10 injected mice died before the end of the study. Surviving mice were sacrificed after 2.5 months (the mouse injected with miR-26 and one injected with miR-33) or 5.5 months (the mouse injected with miR-6 and one injected with miR-33) following vector administration for biochemical analysis. Untreated (untr) age-match mice were used as negative control. (A-C) Vector genome copy number (VGCN) measured by qPCR in (A) heart, (B) quadriceps and (C) liver. VGCN were normalized for the number of copies of titin per sample and expressed as copies per diploid genome. The number of surviving animals used in each presented analysis is indicated in the graphs. (D-E) GYS1 expression analysis by Western Blot, performed on lysates of (D) heart and (E) quadriceps (50µg of total proteins). Quantification of the bands intensity is plotted on the top. GYS1 expression was normalized by GAPDH or Tubulin expression as indicated and expressed relative to the untr. values. (F) GYS1 and GYS2 expression analysis by Western Blot, performed on liver lysates (50µg of total proteins). Quantification of the GYS2 bands intensity is plotted on the top. GYS2 expression was normalized by Tubulin expression and expressed relative to the untr. values. (G-H) Determination of the glycogen content present in the (G) heart and (H) quadriceps lysates. Individual values are plotted.

To finish, glycogen content analysis in the tissues of treated GSD II mice showed a reduction in glycogen accumulation in 3 out of 4 mice, achieving near 60% in the heart and 50% in the quadriceps after the miR-6 delivery (**Figure 3.4G-H**). The correction levels obtained with the other miRNA treatments were more variable with a reduction of glycogen content between 0 and 20% in the heart and between 0 and 40% in the quadriceps compared to untreated GSD II mice (**Figure 3.4G-H**). This partial correction of the glycogen accumulation seems to indicate that a very powerful silencing of GYS1, or even its complete extinction, is required to reduce glycogen accumulation in mice suffering for GSD II.

Similarly, after AAV-mediated genome editing using CRISPR/Cas9 tools and *Gys1*-specific guide RNA, the reduction of GYS1 protein level reached 87% in the heart of treated mice, independently of the promoter used to express the Cas9 (**Figure 3.5A**). The results obtained in sketelal muscle suggest less efficacy of genome editing, with a reduction of GYS1 protein level around 30-40% observed in qaudriceps of treated mice (**Figure 3.5B**). Glycogen content analysis revealed a complete correction of glycogen accumulation in the heart of mice treated with genome editing, with indistinguishable glycogen levels from that of normal mice (**Figure 3.5C**). In the quadriceps, a slight reduction of glycogen accumulation was observed only in the cohort treated with genome editing using a Cas9 expressed under the control of the muscle-specific promoter (**Figure 3.5D**).

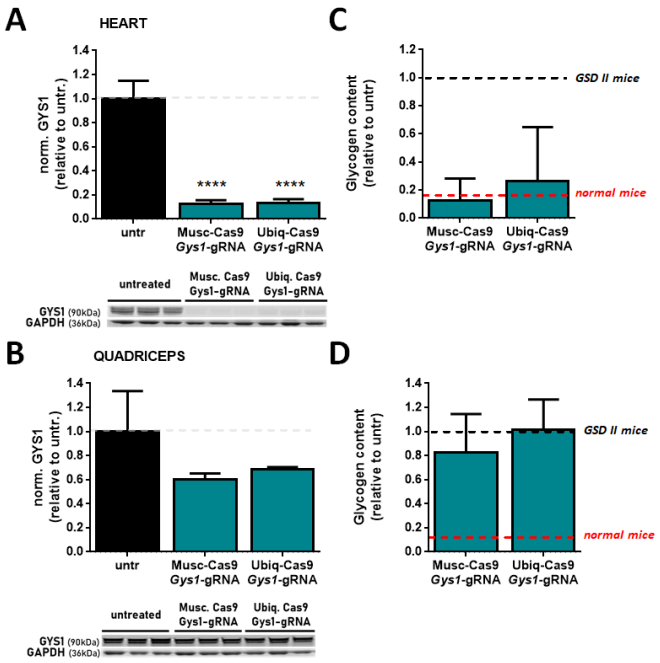

Figure 3.5 Cripr/Cas9-mediated reduction of GYS1 expression prevents glycogen accumulation of GSD II mice
Neonates Gaa$^{-/-}$ mice (0-2 days, n=3 per group) were treated intravenously with 1.5x10^{11}vg/mouse of AAV vectors expressing Gys1-targeting gRNA and the saCas9 under the control of a muscle-specific promoter (Musc.Cas9) or an ubiquitous promoter (Ubiq.Cas9). These mice were sacrificed 6 months after vector administration for biochemical analysis. Untreated (untr) age-match mice were used as negative control. (A-B) GYS1 expression analysis by Western Blot, performed on lysates of (A) heart and (B) quadriceps (50µg of total proteins). Quantification of the bands intensity is plotted on the top. GYS1 expression was normalized by GAPDH expression as indicated and expressed relative to the untr. values. (C-D) Determination of the glycogen content present in the (C) heart and (D) quadriceps lysates. Data are represented as mean ± sd. Statistical analysis were performed by Anova (= p<0.05).*

Together, these results indicate that artificial miRNAs as well as genome editing are efficient approaches to silence GYS1 and decrease glycogen synthesis in heart and skeletal muscle of GSD II mice. Therapeutic efficacy still needs to be improved, specially in skeletal muscle, but this work suggest that GYS1 silencing in neonate mice can prevent the glycogen accumulation and correct the disease phenotype of these mice.

4. Discussion - conclusion

PD (or GSD II) is an inherited metabolic disorder and a progressive neuromuscular disease. It is characterized by a lysosomal α-glucosidase deficiency, resulting in massive glycogen storage in muscle cells, responsible of tissue disruption, autophagy block, and metabolic impairments.[12] Introduction of ERT with rhGAA represented a major advance in the treatment of PD, being a lifesaving treatment for infantile patients.[13-15] However, ERT has

only a moderate effect in late-onset forms of the disease.[16-20] Thanks to enthusiastic results obtained in preclinical models of PD[21-24] and the excellent safety and efficacy profile in human trials for a variety of diseases,[25-29] AAV-mediated gene delivery represents a promising alternative to ERT. Notably, we demonstrated the full rescue of PD in *Gaa*$^{-/-}$ mice using a secretable form of the GAA enzyme expressed in hepatocytes via AAV vectors.[21, 23] This investigational gene therapy has a great potential and could provide superior therapeutic benefit to patients than ERT, but still presents some limitations. One hurdle is related to the fact that hepatic gene transfer with AAV vectors in neonate animals does not persist at long-term.[7] This is an important challenge to the use of this therapeutic strategy in IOPD patients, in who the normal liver growth would lead to a progressive vector genome dilution and loss of treatment efficacy.

To overcome this restriction, SRT in muscles has been proposed.[3-5, 30] Actually, modulation of muscle glycogen accumulation by reducing the expression of the key enzyme responsible of its biosynthesis represents an interesting approach to reverse the pathology in PD. So far, the use of i) phosphorodiamidate morpholino oligonucleotide (PMO) conjugated to a cell penetrating peptide (GS-PPMO);[3] ii) transgenic mouse model;[4] iii) short hairpin RNA (shRNA)-mediated RNA interference;[5] and more recently iv) antisense oligonucleotides (ASO) technology;[30] were attempted to reduce the muscle-specific glycogen synthase (GYS1) expression. Here we proposed other approaches for *Gys1* gene silencing based on the use of artificial miRNAs and genome editing. These strategies offer some advantages over the previous ones.

First, delivered by AAV vector, they allow for a long-term effect as consequence of AAV genome persistence in the muscle tissue (for artificial miRNA strategy) and introduction of permanent modification in the DNA, also transferred during cell division (for genome editing strategy). Hence, one single injection would be required to achieve a long-term gene silencing with our therapeutic strategies.

Artificial miRNA shuttles were proposed as an alternative to shRNA for their safety profile, as the latter have been associated in previous reports with genotoxicity due to off-target effects, activation of the interferon system and cellular miRNA-related toxicity.[31-34] Artificial miRNAs have also the capacity to be associated with Pol II-based expression systems that allow greater control of tissue specificity.[35] Moreover, the use of GS-PPMO was reported as potentially nephrotoxic[36] making artificial miRNAs a good option for gene expression inhibition.

The efficiency of the artificial miRNAs used in this study was first assessed in C2C12 murine myoblasts. Among 300 predicted miRNAs preselected by bioinformatics tools, 7 lead candidates were designed and screened *in vitro* to identify the most efficacious ones. We have demonstrated that miRNAs designed to target murine *Gys1* mRNA sequence (miR-*Gys1*) allowed for an efficient gene silencing in transfected C2C12 cells.

Then, directly injected in tibialis anterior of adult GSD II mice, AAV9 vectors expressing selected miR-*Gys1* were capable of provoking GYS1 protein reduction by approximately 60% of control. Similar results were also obtained with CRISPR/Cas9-mediated genome editing strategy, after injection of AAV9 expressing saCas9 and *Gys1*-specific gRNA in the gastrocnemius of adult GSD II mice. However, despite this *Gys1* knock-down efficacy, we did not observe any effect on the muscle glycogen content. Two assumptions could explain these results. First, the GYS1 protein reduction, and in consequence the cytoplasmic glycogen synthesis reduction, may be not sufficient to be followed by a decline in the lysosomal glycogen content in muscle cells of GSD II mice. A higher vector dose could

overcome this issue, further decreasing GYS1 protein and activity levels. Nevertheless, this option is far to be ideal, taking in consideration some results demonstrating that excessive knockdown of GYS1 may lead to toxicities, similar to those observed in GSD type 0, which is characterized by an increased risk of cardiac arrest.[37, 38] In view of a potential clinical application of GYS1 modulation for PD, it is important to reduce glycogen storage without inducing unexpected severe adverse effects. The second interpretation of the absence of glycogen reduction following GYS1 inhibition, could be the inability of the GSD II mice to degrade lysosomal glycogen accumulated from birth, due to the inherited deficiency in Gaa, and that, regardless of an efficient inhibition of the cytosolic glycogen synthesis.

Performing further studies in pups, we demonstrated the feasibility of a miRNA-based SRT strategy for preventing glycogen accumulation in GSD II mice. The AAV9miR-*Gys1*-mediated knock down of *Gys1* mRNA greatly reduced the amount of GYS1 protein in heart (70% reduction) and skeletal muscle (95% reduction) of GSD II mice treated as neonates. Moreover, no reduction in the GYS2 protein level was observed in the liver of these mice after systemic AAV administration, confirming the specificity of designed miRNAs toward *Gys1* mRNA. We also assessed the effects of miR-*Gys1* treatment on glycogen build up in these GSD II mice. Analysis of both heart and quadriceps revealed modest but obvious declines in glycogen compared to untreated GSD II mice. With the most efficacious artificial miRNA, a respective 60 and 50% reduction was observed in these tissues.

Unfortunately, these encouraging results have to be further confirmed with a larger number of animals, because besides efficacy, this treatment was accompanied by a significant mortality. Given that no apparent signs of progressive decrease in health status were observed, we suspected cardiac arrest linked to excessive knockdown of GYS1. Regrettably, no post-mortem analysis could be performed, making impossible the confirmation of this hypothesis. On the other hand, this was probably not the only possible explanation regarding the results that we obtained in genome editing-treated mice. Indeed, a near complete GYS1 inhibition (98% reduction) was induced in the heart of treated mice following systemic administration of AAV9 expressing saCas9 and *Gys1*-specific gRNA, and no mortality or sign of cardiac toxicity was observed. In addition, although the miR-*Gys1* design has been carefully optimized for specificity, one cannot totally rule out potential unspecific knock down of other mRNAs with sequence similarities. Another important point that we cannot forget at this level of thinking, is the presence of GFP in the expression cassette of AAV9-mir-*Gys1* vectors. At the high AAV vector dose that was used (~$1x10^{14}$vg/kg), the expression of this immunogenic transgene[39] may have resulted in severe adverse effects in these fragile *Gaa*$^{-/-}$ mice. One weakness of our study was the initial small number of animals included per cohort, leaving too few surviving animals for a robust study and concluding results.

Apart from this major shortcoming of the current study, it was very interesting to see, associated with the near total knock down of GYS1, a complete abatement of glycogen build up in the hearts of GSD II mice treated as neonates with CRISPR/Cas9-mediated genome editing. In contrast, no efficacy was observed in quadriceps of those mice, independently of the promoter used to control the Cas9 expression. Further studies would help to better understand the mechanisitic difference, between heart and skeletal muscles tissues in glycogen accumualtion and clearance.
The aim of this study was to demonstrate the feasibility of a miRNA-based and genome editing-based SRT in PD using an AAV-mediated gene transfer approach. Together, the results obtained have evidenced efficacy of these strategies to prevent glycogen build up in GSD II mice treated as neonates. Together, the results corroborate the idea that a very strong GYS1 inhibition (at least > 95%) is required to normalize the glycogen content in GSD II

mice. Further investigations, in larger animal cohorts, need to be performed to confirm these findings and to understand the unexpected mortality that was associated with the miRNA treatments. Without increasing the already high vector dose, other AAV capsids and optimized expression cassettes could be explored for muscle targeting improvement and efficacy enhancement. These approaches could be also developed for a combined muscles and central nervous system targeting for improving the neurological symptoms of the disease. Finally, these SRT strategies could be used in combination with ERT or gene replacement therapy to improve benefits for the PD patients.

In conclusion, our results confirm that SRT strategy may become a future thepeutic strategy for PD.

5. References

1. Raben, N. et al. Targeted disruption of the acid alpha-glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen storage disease type II. *J Biol Chem* **273**, 19086-92 (1998).
2. Bijvoet, A.G. et al. Generalized glycogen storage and cardiomegaly in a knockout mouse model of Pompe disease. *Human molecular genetics* **7**, 53-62 (1998).
3. Clayton, N.P. et al. Antisense Oligonucleotide-mediated Suppression of Muscle Glycogen Synthase 1 Synthesis as an Approach for Substrate Reduction Therapy of Pompe Disease. *Mol Ther Nucleic Acids* **3**, e206 (2014).
4. Douillard-Guilloux, G. et al. Restoration of muscle functionality by genetic suppression of glycogen synthesis in a murine model of Pompe disease. *Hum Mol Genet* **19**, 684-96 (2010).
5. Douillard-Guilloux, G. et al. Modulation of glycogen synthesis by RNA interference: towards a new therapeutic approach for glycogenosis type II. *Hum Mol Genet* **17**, 3876-86 (2008).
6. Ayuso, E., Mingozzi, F. & Bosch, F. Production, purification and characterization of adeno-associated vectors. *Curr Gene Ther* **10**, 423-36 (2010).
7. Bortolussi, G. et al. Life-long correction of hyperbilirubinemia with a neonatal liver-specific AAV-mediated gene transfer in a lethal mouse model of Crigler-Najjar Syndrome. *Hum Gene Ther* **25**, 844-55 (2014).
8. Brinkman, E.K. & van Steensel, B. in CRISPR Gene Editing: Methods and Protocols (ed. Luo, Y.) 29-44 (Springer New York, New York, NY, 2019).
9. Lai, E.C. Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. *Nature genetics* **30**, 363-364 (2002).
10. Lagos-Quintana, M. et al. Identification of tissue-specific microRNAs from mouse. *Current biology* **12**, 735-739 (2002).
11. Raben, N., Nagaraju, K., Lee, E. & Plotz, P. Modulation of disease severity in mice with targeted disruption of the acid α-glucosidase gene. *Neuromuscular Disorders* **10**, 283-291 (2000).
12. van der Ploeg, A.T. & Reuser, A.J. Pompe's disease. *The lancet* **372**, 1342-1353 (2008).
13. Ebbink, B.J. et al. Cognitive decline in classic infantile Pompe disease: An underacknowledged challenge. *Neurology* **86**, 1260-1261 (2016).
14. Parini, R. et al. Long term clinical history of an Italian cohort of infantile onset Pompe disease treated with enzyme replacement therapy. *Orphanet journal of rare diseases* **13**, 32 (2018).
15. Prater, S.N. et al. The emerging phenotype of long-term survivors with infantile Pompe disease. *Genetics in medicine* **14**, 800-810 (2012).
16. Schoser, B. et al. Survival and long-term outcomes in late-onset Pompe disease following alglucosidase alfa treatment: a systematic review and meta-analysis. *Journal of neurology* **264**, 621-630 (2017).
17. Van der Ploeg, A.T. et al. Open-label extension study following the Late-Onset Treatment Study (LOTS) of alglucosidase alfa. *Molecular genetics and metabolism* **107**, 456-461 (2012).
18. Park, J.-S., Kim, H.-G., Shin, J.-H., Choi, Y.-C. & Kim, D.-S. Effect of enzyme replacement therapy in late onset Pompe disease: open pilot study of 48 weeks follow-up. *Neurological Sciences* **36**, 599-605 (2015).
19. Papadopoulos, C. et al. Effect of enzyme replacement therapy with alglucosidase alfa (Myozyme®) in 12 patients with advanced late-onset Pompe disease. *Molecular genetics and metabolism* **122**, 80-85 (2017).
20. Orlikowski, D. et al. Recombinant human acid alpha-glucosidase (rhGAA) in adult patients with severe respiratory failure due to Pompe disease. *Neuromuscular Disorders* **21**, 477-482 (2011).

21. Puzzo, F. et al. Rescue of Pompe disease in mice by AAV-mediated liver delivery of secretable acid alpha-glucosidase. *Sci Transl Med* **9** (2017).
22. Colella, P. et al. AAV Gene Transfer with Tandem Promoter Design Prevents Anti-transgene Immunity and Provides Persistent Efficacy in Neonate Pompe Mice. *Mol Ther Methods Clin Dev* **12**, 85-101 (2019).
23. Cagin, U. et al. Rescue of advanced Pompe disease in mice with hepatic expression of secretable acid α-glucosidase. *Molecular Therapy* (2020).
24. Ronzitti, G., Collaud, F., Laforet, P. & Mingozzi, F. Progress and challenges of gene therapy for Pompe disease. *Ann Transl Med* **7**, 287 (2019).
25. Mendell, J.R. et al. Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. *N Engl J Med* **377**, 1713-1722 (2017).
26. George, L.A. et al. Hemophilia B Gene Therapy with a High-Specific-Activity Factor IX Variant. *N Engl J Med* **377**, 2215-2227 (2017).
27. Nathwani, A.C. et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* **371**, 1994-2004 (2014).
28. Bennett, J. et al. Safety and durability of effect of contralateral-eye administration of AAV2 gene therapy in patients with childhood-onset blindness caused by RPE65 mutations: a follow-on phase 1 trial. *Lancet* **388**, 661-72 (2016).
29. Bainbridge, J.W. et al. Long-term effect of gene therapy on Leber's congenital amaurosis. *New England Journal of Medicine* **372**, 1887-1897 (2015).
30. Kimonis, V. et al. Antisense oligonucleotide treatment targeting glycogen synthase (GYS1) in a mouse model of Pompe disease. *Molecular Genetics and Metabolism* **126**, S85 (2019).
31. Sledz, C.A., Holko, M., De Veer, M.J., Silverman, R.H. & Williams, B.R. Activation of the interferon system by short-interfering RNAs. *Nature cell biology* **5**, 834-839 (2003).
32. Borel, F. et al. In vivo knock-down of multidrug resistance transporters ABCC1 and ABCC2 by AAV-delivered shRNAs and by artificial miRNAs. *Journal of RNAi and gene silencing: an international journal of RNA and gene targeting research* **7**, 434 (2011).
33. Grimm, D. et al. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**, 537-541 (2006).
34. Boudreau, R.L., Martins, I. & Davidson, B.L. Artificial microRNAs as siRNA shuttles: improved safety as compared to shRNAs in vitro and in vivo. *Molecular Therapy* **17**, 169-175 (2009).
35. Wiznerowicz, M., Szulc, J. & Trono, D. Tuning silence: conditional systems for RNA interference. *Nature methods* **3**, 682-688 (2006).
36. Moulton, H.M. & Moulton, J.D. Morpholinos and their peptide conjugates: therapeutic promise and challenge for Duchenne muscular dystrophy. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1798**, 2296-2303 (2010).
37. Sukigara, S. et al. Muscle glycogen storage disease 0 presenting recurrent syncope with weakness and myalgia. *Neuromuscular Disorders* **22**, 162-165 (2012).
38. Kollberg, G. et al. Cardiomyopathy and exercise intolerance in muscle glycogen storage disease 0. *New England Journal of Medicine* **357**, 1507-1514 (2007).
39. Ansari, A.M. et al. Cellular GFP toxicity and immunogenicity: potential confounders in in vivo cell tracking experiments. *Stem cell reviews and reports* **12**, 553-559 (2016).

Chapter 4

Innovative AAV vectors to improve muscle targeting

Unpublished data

1. Introduction

Gene transfer targeting the muscle tissues, including skeletal muscle, heart, and diaphragm, is one of the most important approaches for Pompe Disease (PD), due to the central role of these tisues in the pathophysiology of the disease, and can be applied for both infantile-onset PD (IOPD) and late-onset PD (LOPD) patients.

Among the most promising vectors for muscle-directed gene therapy, AAV vectors represent the most attractive. Over the past two decades, different AAV serotypes have been explored to develop muscle-gene transfer strategies to treat PD, including AAV1[1-4], AAV2[5], AAV5[6, 7], AAV6[8], AAV8[7, 9-12] and AAV9[4, 13-18]. To drive the therapeutic transgene expression, the cytomegalovirus (CMV) promoter and the muscle-specific desmin promoter have been the most commonly used in these studies. However, the vectors developed so far, although interesting in animal models of PD,[1, 8, 10, 19] require high vector doses to achieve widespread muscle transduction, not easily achievable when addressing the LOPD patients population. Clinical use of such high vector doses may be hazardous owing to possible immuno/hepato-toxicity in humans.[20-24]

The same statement was also made for other diseases, such as in Duchenne muscular dystrophy (DMD) or in X-linked myotubular myopathy (XLMTM), where it has been particularly challenging to achieve robust expression of the therapeutic gene in the skeletal muscle.[25-31] Thus, it seems essential to develop more potent muscle-specific gene therapy vectors containing novel regulatory elements that outperform the conventional ones (i.e. CMV,[32, 33] desmin,[34-36] etc.) and allow for high muscle-specific expression at lower and safer vector doses.

Generation of synthetic muscle-specific promoter, such as SPc5-12, has been proposed to boost muscle-specific gene expression but has resulted in a relatively modest increase in muscle-specific expression relative to the CMV promoter.[26, 37-39] Today, in an effort to overcome this bottleneck that hampers muscle-directed gene therapy, construction of combined compact muscle-specific promoters is an attractive alternative.[40] Here, we tested next-generation AAV vectors, specifically designed for PD. This innovative myospecific platform technology was focused on boosting gene transfer efficiency specifically in the muscle, and increasing expression by using more robust muscle-specific expression cassettes. To test that, we explored the efficacy of the newly developed AAV vectors in reducing the pathological glycogen accumulation in GSDII mouse models.

Overall, our data suggest that the PD-specific AAV prototypes bring enhanced muscle-specific gene expression and delivery and offer the prospect of safer and more effective vectors for myopathies. Additional investigations will allow to confirm these encouraging preliminary results and further development will help to reduce the humoral response directed against human GAA.

2. Materials and methods

2.1 Test Items: GAA expression cassettes and AAV vectors

Test items were generated in the frame of the European consortium Myocure (supported by the European Union's research and innovation program under grant agreement nos. 667751)

GAA transgene expression cassettes containing the wild-type (GAAwt) or codon-optimized (GAAco) coding sequences encoding for the native form of human GAA were designed to maximize muscle-specific expression and were kindly provided by the group of Dr. Marinee K.L. Chuah and Prof. Thierry VandenDriessche (University of Brussels, Belgium). Briefly, GAA coding sequences were cloned in an AAV vector backbone containing the wild-type AAV2 ITRs and a synthetic polyA signal, and placed under control of the SpC5-12 promoter or a fusion of the SpC5-12 promoter and the innovative de novo designed enhancer CRE04-CSkSH5. This regulatory element is a combination of the most robust myospecific-cis regulatory modules (CRMs), identified and validated by computational vector design.[41]

The group of Prof. Fatima Bosch (Autonomous University of Barcelona, Spain) has produced, purified and characterized the research-grade preparations of AAV vectors. The capsid sequences (AAV9, AAVS1P1 and AAVS10P1) were developed and kindly provided by the group of Prof. Dirk Grimm (Heidelberg University Hospital, Germany).

2.2 *In vivo* studies

Mice

Mouse studies were performed according to the French and European legislation on animal care and experimentation (2010/63/EU) and approved by the local Institutional Ethical Board (protocols no. 2015-008 and 2017-011B, apafis no. 13643). Mice were kept in a temperature-controlled environment with 12/12-hour light–dark cycle. They received a standard chow diet and water ad libitum.

The *Gaa*$^{-/-}$ mice were purchased from the Jackson Laboratory (B6;129-Gaa^{tm1Rabn}/J, stock no. 004154, 6neo). This mouse model was originally generated Raben et al.[42] *Gaa*$^{+/+}$ mice are derived from the same colony and breed separately. CD4-deficient Pompe disease mice were generated by successive crossings of *Gaa*$^{-/-}$ and *Cd4*$^{-/-}$ mice. Genotypes were confirmed by polymerase chain reaction (PCR) performed on genomic DNA with oligonucleotides specific for the mutated regions in each gene.

Gaa (mutant)	forward 5'-CGTTGGCTACCCGTGATATT-3'
Gaa (wild-type)	forward 5'-TCCTGAGCCCAAACACTTCT-3'
Gaa	reverse 5'-ATTGTTGCACAACGCTCTTG-3'
Cd4 (mutant)	forward 5'-GTGTTGGGTCGTTTGTTCG-3'
Cd4 (wild-type)	forward 5'-CCTCTTGGTTAATGGGGGAT-3'
Cd4	reverse 5'-TTTTTCTGGTCCAGGGTCAC-3'

Gene transfer procedures

Three-four months old *Gaa*$^{-/-}$ mice, were injected in the tail vein with 1x10^{13}vg/kg of AAV vector serotype 9 (AAV9), S1P1 (AAVS1P1) or S10P1 (AAVS10P1) expressing human GAA (GAAwt). In additional groups, a codon optimized version of GAA was used (GAAco). GAA transgene expression was driven by SPc5-12 promoter, or by a fusion SPc5-12 promoter / CRE04-CSkSH5 enhancer (Enh-GAAco). PBS-injected Gaa$^{+/+}$ and Gaa$^{-/-}$ mice were used as controls (WT and KO respectively); n= 3-4 per group. Antibody measurements were performed at two weeks after vector injection and then monthly. Three

months after vector injection mice were sacrificed and tissues collected to evaluate the biochemical correction.

Three-four months old *Cd4*$^{-/-}$ *Gaa*$^{-/-}$ mice, were injected in the tail vein with $3x10^{12}$vg/kg to $1.6x10^{13}$vg/kg of AAV vector serotype 9 (AAV9), S1P1 (AAVS1P1) or S10P1 (AAVS10P1) expressing human GAA (GAAwt) under the control of the SPc5-12 promoter or expressing a codon optimized (co) version of GAA (GAAco) driven by a fusion SPc5-12 promoter / CRE04-CSkSH5 enhancer (Enh-GAAco). PBS-injected Gaa$^{+/+}$ and Gaa$^{-/-}$ mice were used as controls (WT and KO respectively).; n= 3-4 per group. Three months after vector injection mice were sacrificed and tissues collected to evaluate the biochemical correction.

GAA Activity Assay

Snap-frozen tissues were homogenized in UltraPure DNase- and RNase-free distilled water (Thermo Fisher Scientific). Tissues were weighed, homogenized, and centrifuged for 10 min at 10,000×g to collect the supernatant. The enzymatic reaction was set up using 10µL of tissue homogenate, diluted appropriately and 20µL of substrate, 4-methylumbelliferone (4MU)α-D-glucoside, in black 96-well plates (PerkinElmer). The reaction mixture was incubated at 37°C for 1hr and then stopped by adding 150µL of sodium carbonate buffer (pH 10.5). A standard curve (0–2,500pmol/µL of 4MU) was used to measure released fluorescent 4MU from the individual reaction mixture using the EnSpire Alpha plate reader (PerkinElmer) at 449 nm (emission) and 360 nm (excitation). The protein concentration of the clarified supernatant was quantified by BCA (Thermo Fisher Scientific). To calculate the GAA activity in tissues, the released 4MU concentration was divided by the sample protein concentration, and activity was reported as nmol/hr/mg protein.

Western blot analysis

Total proteins concentration of tissue homogenates, prepared as described above, was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) following manufacturer instructions and protein extracts were separated on a 4-12% bis-tris polyacrylamide gel (Thermo Fisher Scientific, Waltham, MA). The same amount of protein was loaded in each lane. The gel was transferred onto a nitrocellulose membrane and blotted with anti-GAA antibody (AbcamCambridge, UK). Anti-GAPDH antibody (Thermo Fisher Scientific, Waltham, MA) was used as loading control. Secondary antibodies and detection system were from Li-Cor Biosciences (Lincoln, NE).

Glycogen content measurement

Glycogen content was analyzed in tissue homogenates, prepared as previously described. 20µl of homogenate properly diluted was mixed with 55µl of distilled water, incubated for 5min at 95°C, and cooled at 4°C. Next, glycogen contained in samples was digested with amyloglucosidase from Aspergillus Niger (Sigma Aldrich, Saint-Louis, MO), in order to release the glucose monomers. For that, 25µl of amyloglucosidase diluted 1:50 in 0.1M potassium acetate pH5.5 was added to each sample. A control reaction without amyloglucosidase was done in parallel for each sample. Both digestion and control samples were incubated at 37°C for 90min, followed by 5min at 95°C. Glycogen content was determined indirectly by quantifying the number of glucose molecules released per gram of

protein, with the use of a colorimetric glucose assay kit (Sigma Aldrich, Saint-Louis, MO). Glucose assay was performed according to the manufacturer's instructions. Absorbance was measured with an EnSpire alpha plate reader (PerkinElmer) at 540 nm.

Anti-GAA antibody Detection

Anti-hGAA IgG capture assays were performed as described previously.[9] Briefly, Maxisorp 96-well plates (Thermo Fisher Scientific) were coated with 2µg/mL of rhGAA. IgG standard curves were made by serial 1 to 2 dilution of commercial mouse recombinant IgGs (Sigma-Aldrich) that were coated directly onto the wells in duplicate (from 1 µg/mL to 0.15 µg/mL). Plasma samples appropriately diluted in 10mM PBS (pH 7.4) containing 2% BSA were analyzed in duplicate. An HRP-conjugated anti-mouse IgG antibody (human ads-HRP, Southern Biotech) was used as a secondary antibody. Plates were revealed with OPD substrate (o-phenylenediaminedihydrochloride, Sigma). The reaction was stopped with H2SO4 3M solution, and optical density (OD) measurements were done at 492 nm using a microplate reader (ENSPIRE, PerkinElmer, Waltham, USA). Anti-hGAA IgG concentration was determined against the standard curve.

Statistical analysis

Results are expressed as mean ± SD as described in the text. The Prism package (Graph Pad Software, La Jolla, CA) was used to analyze data. Statistical analysis has been performed by one or two way ANOVA as described in the text. Values of $p < 0.05$ were considered as statistically significant.

3. Results

To establish an effective cure for PD, robust expression of the GAA gene in the heart, skeletal muscle and diaphragm is required, since patients suffer from progressive muscle weakness and frequently die from cardio-respiratory failure. A validated computational approach,[43-45] was used to identify robust cis-regulatory modules (CRMs) capable of substantially increasing transcription in those tissues at the University of Leuven. This required 4 consecutive computational steps: (1) highly expressed myospecific genes were identified based on statistical analysis of micro-array expression data obtained with normal human tissues; (2) publicly available databases were used to extract the corresponding promoter sequences; (3) a computational approach was employed to identify clusters of transcription factor binding site motifs (TFBS); and (4) the genomic context of the highly expressed genes was screened for evolutionary conserved clusters of TFBS (i.e. CRMs). Then, the optimal *de novo* designed muscle-derived CRM was cloned upstream of the SpC5-12 promoter, to drive GAA expression in muscle tissues.

In order to increase muscle-specific gene delivery, new muscle-tropic AAV capsids were developed by employing peptide display and DNA shuffling technologies. *In vivo* screening of barcoded libraries and next-generation sequencing resulted in several highly promising AAV capsids and peptides that efficiently transduce the three on-targets (heart, skeletal muscle and diaphragm) while exhibiting reduced activity in off target tissues (e.g. liver, spleen, lungs, kidney, brain, intestines, immune cells, etc.), such as new chimeras AAVS1 and AAVS10 (derived from AAV1-6-8-9-po1 shuffled libraries) and the peptide RGDLGLS called P1.

Consequently, these prototypes of AAV vectors, designed for an optimal muscle gene transfer specifically in PD, were generated expressing GAA coding sequences driven from the most optimal *de novo* designed muscle-derived CRMs. This next-generation muscle-directed gene therapy platform is summarized in **Figure 4.1**.

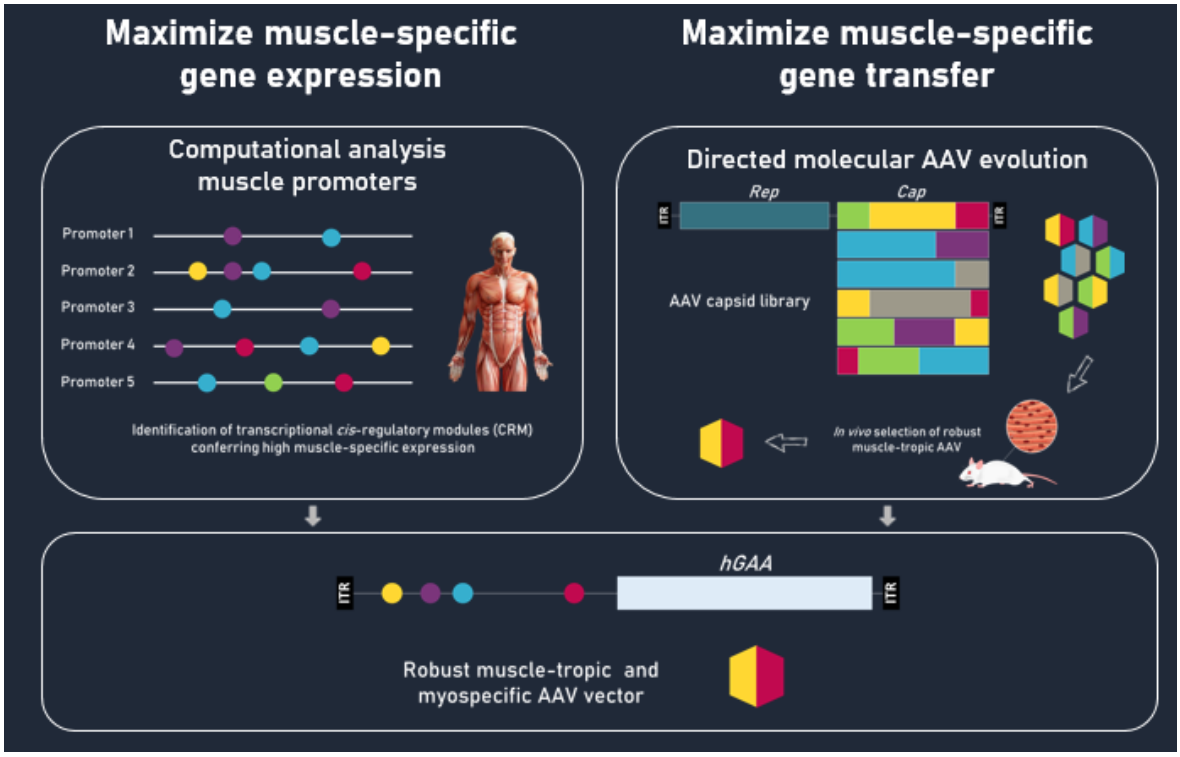

Figure 4.1 Next-generation muscle-directed gene therapy by in silico vector design

3.1 Novel AAV capsids transduced efficiently muscles in GSD II mice

To further validate the muscle-specific tropism of the newly developed capsids, an efficacy study was performed in adult GSD II mice (**Figure 4.2**). Animals were intravenously injected with $1x10^{13}$vg/kg of AAV9, AAVS1P1 or AAVS10P1 vectors expressing native human GAA under the control of the Sp5-12 promoter. Three months after AAV vectors injection, the mice were sacrificed and tissues harvested to evaluate the biochemical correction achieved by the treatment. Biodistribution analysis (data not shown) confirmed the preferential muscle tropism of the capsids with the presence of AAV vector genomes detected in the muscle tissues (heart, diaphragm and skeletal muscles) in all AAV treated cohorts, but not in the livers of those animals, at the exception of the AAV9 treated group.

Next we quantified the hGAA protein expression levels in the different tissues of the injected GSD II mice. Three months after treatment, western blot analyses of heart lysates showed high amount of hGAA in all the GSD II mice treated with AAV vectors, regardless of the capsid injected (**Figure 3.2A**). In diaphragm and triceps, the highest hGAA expression levels were observed in animals who received the new capsids (AAVS1P1 and AAVS10P1) (**Figure 4.2B-C)**. Especially, mice receiving the AAVS1P1 vector showed a significantly increased hGAA expression in those tissues when compared to AAV9-injected animals. In addition, the specificity of the gene expression and delivery in the muscles of the two new

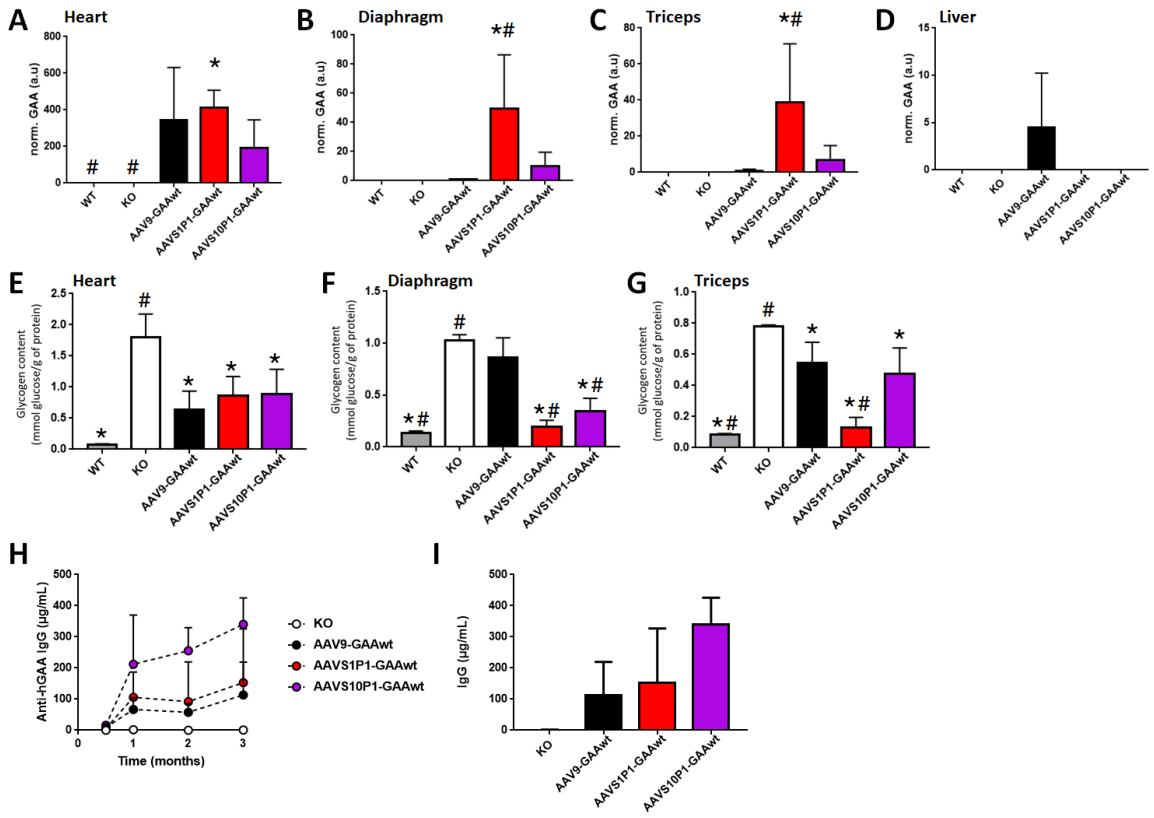

Figure 4.2 Enhancement of muscle gene delivery in GSD II mice with AAVS1P1 and AAVS10P1 vectors
$Gaa^{-/-}$ mice were injected with $1x10^{13}$ vg/kg of AAV vector serotype 9 (AAV9-GAAwt), S1P1 (AAVS1P1-GAAwt) or S10P1 (AAVS10P1-GAAwt) expressing human GAA. In all groups, GAA transgene expression was driven by SPc5-12 promoter. PBS-injected $Gaa^{+/+}$ and $Gaa^{-/-}$ mice were used as controls (WT and KO respectively). n=4 per group at vector injection. Antibody measurements were performed at two weeks after vector injection and then monthly. Three months after vector injection mice were sacrificed and tissues collected to evaluate the biochemical correction (A-D) GAA expression in tissues. Human GAA levels were quantified by western blot in heart (A), diaphragm (B), triceps (C) and liver (D) of treated animals. GAA expression was normalized by GAPDH expression and expressed in arbitrary unit (a.u). Results are represented by mean ± SD. Statistical analyses were performed by ANOVA (= p<0.05 vs PBS treated $Gaa^{-/-}$ mice, # = p<0.05 vs AAV9-GAAwt treated $Gaa^{-/-}$ mice; n=3-4 mice per group). (E-G) Glycogen accumulation in tissues. Glycogen levels were measured in heart (E), diaphragm (F), and triceps (G) of treated animals. Results are represented by mean ± SD. Statistical analyses were performed by ANOVA (* = p<0.05 vs PBS treated $Gaa^{-/-}$ mice, # = p<0.05 vs AAV9-GAAwt treated $Gaa^{-/-}$ mice; n=3-4 mice per group). (H-I) Antibody measurements. (H) Time course of anti-GAA antibodies formation in immunocompetent $Gaa^{-/-}$ mice, measured by ELISA. (I) Levels of anti-GAA binding IgG measured at 3 months post injection. Results are represented by mean ± SD and are expressed as µg of IgG per µL of serum measured by using an IgG standard curve coated in parallel with the samples. Statistical analyses were performed by two-way ANOVA (* = p<0.05 vs PBS treated Gaa-/- mice, n=3-4 mice per group).*

capsids was confirmed by the lack of hGAA expression in non-target tissues, such as the liver (**Figure 4.2D**).

Then we measured the glycogen content in the muscles of the GSD II mice. Three months after treatment, a general decrease of pathological glycogen accumulation was observed in AAV-treated GSD II mice compared with those treated with PBS (KO) (**Figure 4.2E-G**). In diaphragm (**Figure 4.2F**) only AAVS1P1 and AAVS10P1 vectors but not AAV9, led to normalization of glycogen accumulation. In triceps (**Figure 4.2G**), only AAVS1P1 vector led to a complete correction of glycogen build up with levels undistinguishable from those measured in wild-type animals (WT). However, at the end of the study, no rescue of the muscle strength was observed in AAV-treated animals, regardless of the capsid used. (data not shown).

Gene transfer of native hGAA to *Gaa*$^{-/-}$ mice driven by either ubiquitous or muscle-specific promoters has been reported to induce unwanted humoral immune responses toward the hGAA protein.[8, 9, 18, 19, 46, 47] To assess the immunogenicity of the new AAV prototypes we evaluated the anti-hGAA humoral immune responses in injected mice along the protocol. A time-dependent increase of anti-GAA IgG was observed in all AAV-injected mice (**Figure 4.2H-I**). In the GSD II mice treated with the AAVS10P1 vector, a significant increase of the levels of anti-human GAA IgG was observed at 2 and 3 months post-treatment. On the other hand, similar levels of anti-hGAA IgG were observed in mice treated with AAVS1P1 or AAV9 vectors.

Overall, these data suggest that AAVS1P1 and AAVS10P1 vectors allow improvement of muscle gene transfer efficacy, with a significant increase of hGAA expression and a reduction of the glycogen content in target tissues and especially in diaphragm and skeletal muscles. The capsid AAVS1P1 seem to have a high efficacy in skeletal muscle and to induce a lower immune response against the transgene compared to the AAVS10P1.

3.2 hGAA expression cassette optimizations increase the gene expression

Based on the *in silico* data, we assessed the ability of the optimized hGAA expression cassettes (GAAco and Enh.GAAco) to drive muscle-specific transgene expression following AAVS10P1 gene transfer in GSD II mice (**Figure 4.3**). AAVS10P1 vectors were then generated to express native hGAA (GAA) or codon-optimized hGAA (GAAco) under the control of the SpC5-12 or the newly *de novo* designed fusion of the SpC5-12 promoter with the CRE04-CSkSH5 enhancer (Enh.GAAco).

Three months after intravenous injection of AAV vectors at a dose of $1x10^{13}$vg/kg, liver, cardiac muscle (heart), skeletal muscle and diaphragm were collected to evaluate hGAA expression and glycogen content. In target muscle tissues, although high hGAA expression was observed using all the expression cassettes, the Enh.GAAco was significantly more active than the others (**Figure 4.3A-C**). The lack of hGAA expression in the liver (**Figure 4.3D**) supported the muscle specificity of the gene expression and delivery of the new prototype AAV vectors specifically designed for PD.

Then we measured the glycogen content in the muscles of the GSD II mice. Three months after treatment, pathological glycogen accumulation was significantly reduced in heart (**Figure 4.3E**), diaphragm (**Figure 4.3F**), and triceps (**Figure 4.3G**), of all AAV-treated mice compared with PBS-treated GSD II mice (KO). In triceps, only AAVS10P1-Enh.GAAco vector led to a complete correction of glycogen build up with levels undistinguishable from those measured in wild-type animals (WT) (**Figure 4.3G**). However, at the end of the study, no rescue of the muscle strength was observed in AAV-treated animals, regardless of the expression cassette used (data not shown).

Finally, the anti-GAA humoral response was evaluated in these mice. As previously reported, a time-dependent increase of anti-GAA IgG was observed in all AAVS10P1-injected mice. Similar levels were obtained independently of the hGAA expression cassette employed (**Figure 4.3H-I**).

Together, these data suggest that the optimized hGAA expression cassette, composed by myo-derived-CRMs and the robust SpC512 promoter, specifically optimized for PD, increases the muscle-specific expression of hGAA.

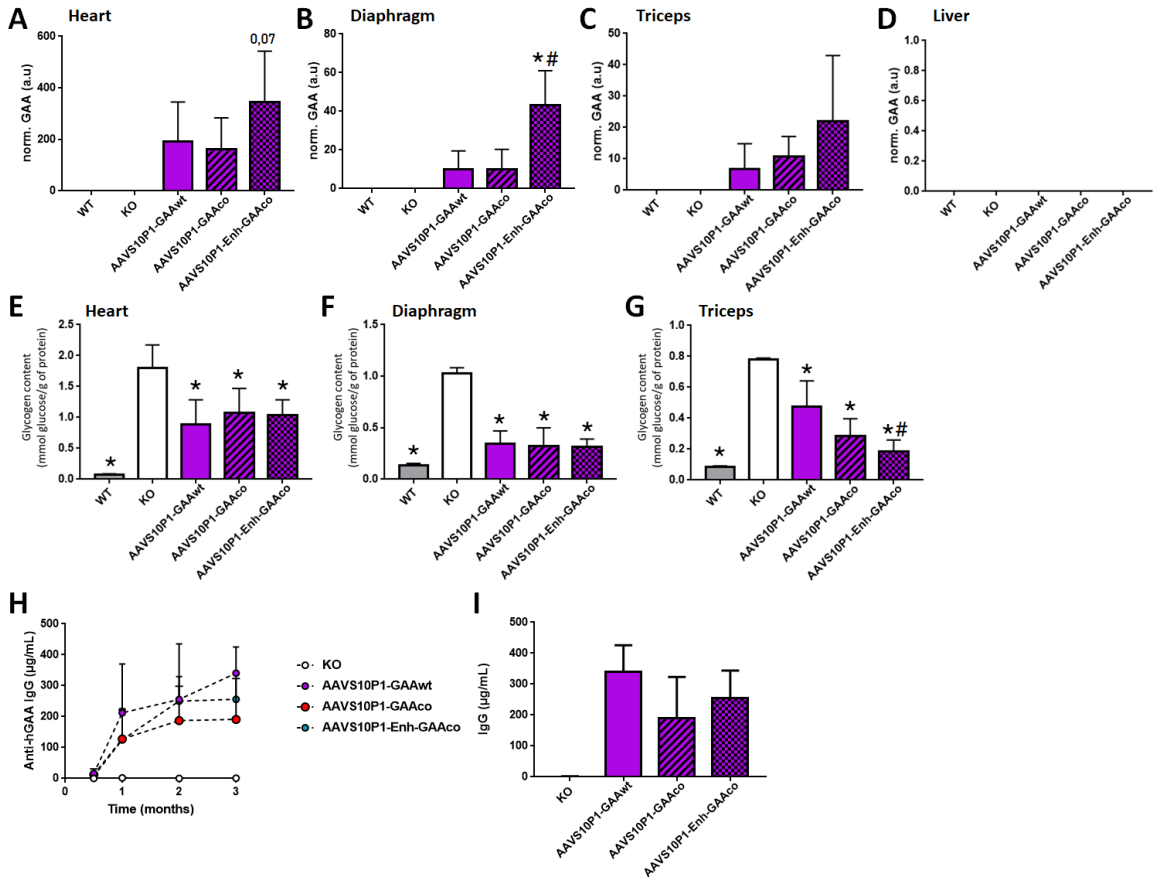

Figure 4.3 In vivo efficacy study (expression cassettes)
$Gaa^{-/-}$ mice were injected with $1x10^{13}$ vg/kg of AAV vector serotype S10P1 expressing human GAA (AAVS10P1-GAAwt) or a codon optimized (co) version of GAA (AAVS10P1-GAAco). In these groups, GAA transgene expression was driven by SPc5-12 promoter. In one additional group, where the CRE04-CSkSH5 enhancer was fused with the SPc5-12 promoter to express the codon optimized version of the human GAA transgene (AAVS10P1-Enh-GAAco). PBS-injected $Gaa^{+/+}$ and $Gaa^{-/-}$ mice were used as controls (WT and KO respectively). n=4 per group at vector injection. Antibody measurements were performed at two weeks after vector injection and then monthly. Three months after vector injection mice were sacrificed and tissues collected to evaluate the biochemical correction (A-D). GAA expression in tissues. Human GAA levels were quantified by western blot in heart (A), diaphragm (B), triceps (C) and liver (D) of treated animals. GAA expression was normalized by GAPDH expression and expressed in arbitrary unit (a.u). Results are represented by mean ± SD. Statistical analyses were performed by ANOVA (= p<0.05 vs PBS treated $Gaa^{-/-}$ mice, # = p<0.05 vs AAV9-GAAwt treated $Gaa^{-/-}$ mice; n=3-4 mice per group). (E-G) Glycogen accumulation in tissues. Glycogen levels were measured in heart (E), diaphragm (F), and triceps (G) of treated animals. Results are represented by mean ± SD. Statistical analyses were performed by ANOVA (* = p<0.05 vs PBS treated $Gaa^{-/-}$ mice, # = p<0.05 vs AAV9-GAAwt treated $Gaa^{-/-}$ mice; n=3-4 mice per group). (H-I) Antibody measurements. (H) Time course of anti-GAA antibodies formation in immunocompetent $Gaa^{-/-}$ mice, measured by ELISA. (I) Levels of anti-GAA binding IgG measured at 3 months post injection. Results are represented by mean ± SD and are expressed as µg of IgG per µL of serum measured by using an IgG standard curve coated in parallel with the samples. Statistical analyses were performed by two-way ANOVA (* = p<0.05 vs PBS treated Gaa-/- mice, n=3-4 mice per group).*

3.3 New AAV vector prototypes correct the glycogen accumulation in GSD II mice

Based on the efficacy studies results and on the formation of anti-transgene antibody response described earlier, a vector dose-response analysis of the new GSD II-specific prototype AAV vector (AAVS1P1-Enh-hGAAco) was performed in *Cd4*/*Gaa* double KO mice, which cannot mount any humoral immune response against hGAA. Vectors doses from $3x10^{12}$ to $1.6x10^{13}$vg/kg were administered intravenously in mice and compared to medium dose of AAV9 or AAVS10P1 vectors. Then, the biochemical correction achieved by the treatments was evaluated three months after vector injection (**Figure 4.4**).

Measurement of GAA activity in muscle (heart, diaphragm, and triceps) showed increased GAA activity in AAV-treated GSD II mice groups compared to PBS-treated mice, irrespective of the vector used (**Figure 4.4A-C**). Especially, supra-physiological GAA activity was achieved following AAV vectors administration in cardiac and skeletal muscle. If the AAV9 treatment at the medium dose ($8x10^{12}$vg/kg) provided the highest activity in the heart (**Figure 4.4A**), the new AAV vector prototypes were the most efficacious vectors to express GAA in the triceps (**Figure 4.4C**). In particular, the GAA activity measured in AAVS1P1-injected cohorts at medium and high doses was significantly higher than in other cohorts. In the other tissues, such as spinal cord (**Figure 4.5A**), liver, lungs or brain no significant GAA activity improvement was observed in AAV-treated groups compared to PBS-injected GSD II mice (data not shown).

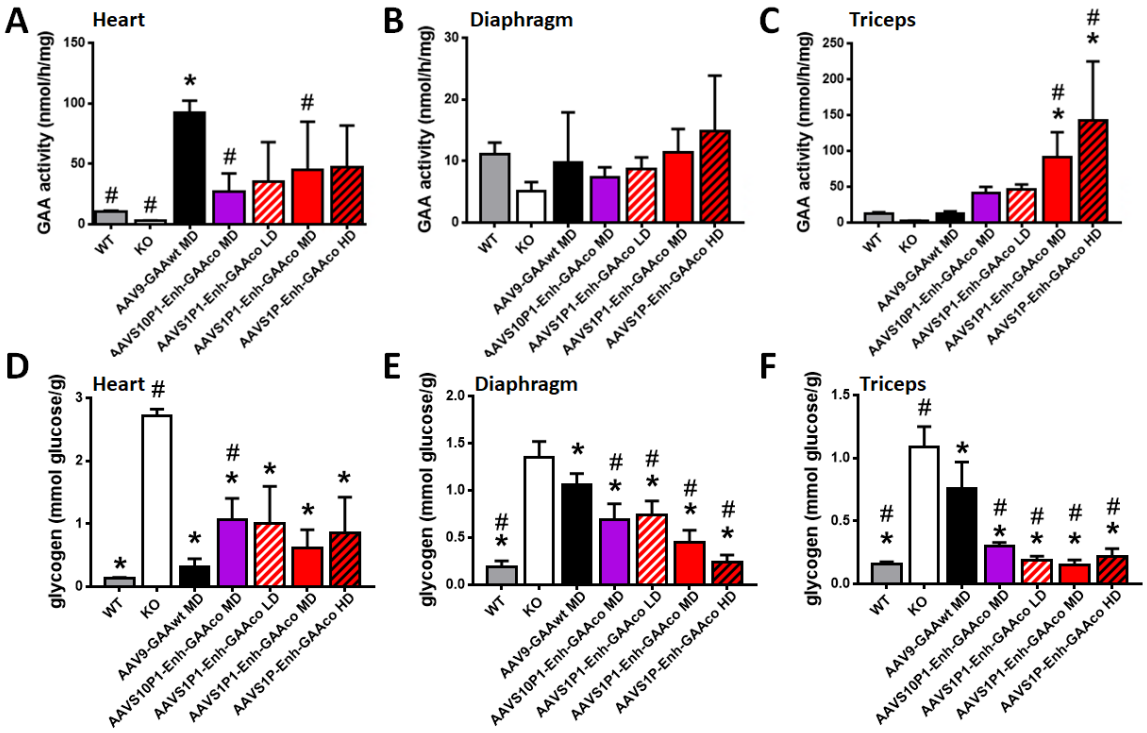

Figure 4.4 Dose-finding study of AAVS1P1-Enh-GAAco vector in GSD II mice
Adult CD4/Gaa double KO mice were treated with AAV vector serotype 9 expressing the wild-type (wt) version of human GAA under the control of the SPc5-12 promoter (AAV9-GAAwt), AAV vector serotype S10P1 (AAVS10P1-Enh-GAAco) or S1P1 (AAVS1P1-Enh-GAAco) expressing a codon optimized (co) version of human GAA driven by the SPc5-12 promoter fused with the CRE04-CSkSH5 enhancer (Enh). Injected vector doses were: $3x10^{12}$vg/kg (low dose, LD), $8x10^{12}$vg/kg (medium dose, MD) or $1.6x10^{13}$vg/kg (high dose, HD). PBS-injected $Gaa^{+/+}$ and $Gaa^{-/-}$ mice were used as controls (WT and KO respectively). Tissues were collected to evaluate the biochemical correction. (A-C) GAA expression in tissues. GAA activity was measured three months after vectors injection in heart (A), diaphragm (B), and triceps (C) of treated animals. (D-F) Glycogen accumulation in tissues. Glycogen levels were measured three months after vectors injection in heart (D), diaphragm (E) and triceps (F) of treated animals. Results are represented by mean ± SD. Statistical analyses were performed by ANOVA (= $p<0.05$ vs PBS treated $Gaa^{-/-}$ mice, # = $p<0.05$ vs AAV9-GAAwt treated $Gaa^{-/-}$ mice; n=3-4 mice per group).*

We evaluated glycogen accumulation in the different tissues of the injected mice. In line with the GAA activity data, an overall decrease of glycogen accumulation was observed in muscles of AAV-treated GSD II mice (**Figure 4.4D-F**). In the cardiac muscle, glycogen levels were significantly decreased in all the cohorts injected with AAV vectors. Notably, in the AAV9-, medium and high doses AAVS1P1-treated cohorts, the glycogen levels were undistinguishable from those measured in PBS-treated GSD II animals (**Figure 4.4D**). In diaphragm, glycogen levels in mice treated with the new GSD II-specific AAV vector prototypes were significantly lower compared to those observed in mice that received

AAV9-GAAwt injection and reached the wild-type levels in medium and high dose AAVS1P1-treated animals (**Figure 4.4E**). In triceps, a general decrease of glycogen levels was observed in all AAV-treated mice and a complete correction of glycogen build-up was achieved in both AAVS10P1 and AAVS1P1-treated groups (**Figure 4.4F**). However, functional evaluation tests performed at two and three months post-injection did not showed any correction of the muscle strength impairment observed in GSD II mice regardless of the vector injected (data not shown).

In liver and as previously noted, glycogen levels were similar in *Gaa*$^{+/+}$ and *Gaa*$^{-/-}$ animals and none of the vectors reduced the glycogen content in this tissue (data not shown). In spinal cord (**Figure 4.5B**), lungs, and brain (data not shown), no correction of the glycogen accumulation was observed in mice treated with AAV vectors.

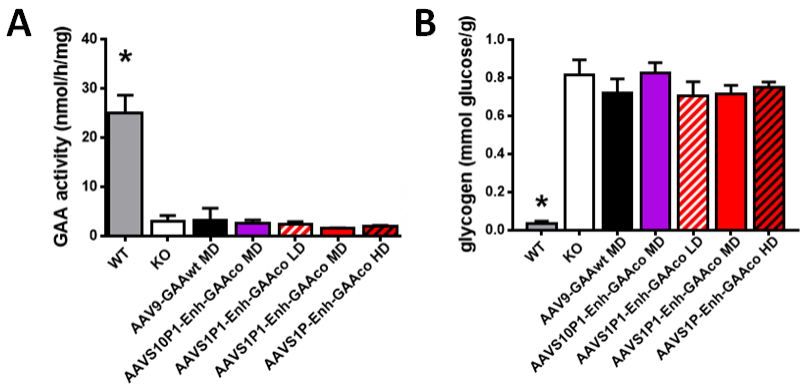

Figure 4.5 Dose-finding study of AAVS1P1-Enh-GAAco vector in GSD II mice: results in central nervous system
Adult CD4/Gaa double KO mice were treated as described in Figure 3.4 GAA activity (A) and glycogen levels (B) were measured three months after vectors injection in spinal cord of treated animals. Results are represented by mean ± SD. Statistical analyses were performed by ANOVA (= p<0.05 vs PBS treated Gaa$^{-/-}$ mice; n=3-4 mice per group).*

Overall, these data demonstrated that both new AAV prototypes, specifically designed for PD, allow improvements of muscle gene transfer efficacy, with a significant increase of the GAA activity and a reduction of the glycogen content in skeletal muscles. Importantly, mice treated with medium and high doses of AAVS1P1-Enh-GAAco showed correction of glycogen accumulation in heart, diaphragm and skeletal muscle.

4. Discussion - conclusion

PD (or GSD II) is a rare inherited metabolic and muscle-wasting disorder for which there is an urgent medical need. Children affected by the most severe form of this disease suffer from progressive skeletal muscle weakness and without medical intervention, can die within their first year of life from cardiorespiratory failure. Current treatment for PD involves the administration of functional acid alpha-glucosidase recombinant enzyme as an injection, approach known as ERT. Introduction of this treatment represented a major advance in the treatment of PD, being a lifesaving treatment for infantile patients,[48-50] however, ERT has only a moderate effect in late-onset forms of the disease.[51-55]

As a response to the limitations of ERT, gene therapy represents a promising alternative. Over the last years, a hope has emerged thanks to the successful implementation and validation of AAV-based gene therapies, including following systemic administration.[1-3, 5,

[7-10, 15, 16, 18, 19, 56-59] Especially, the AAV9 has shown great promise in preclinical evaluation, notably due to its capability to transduce skeletal muscle, diaphragm, and heart, as reported by many labs.[4, 15, 16, 60] It was also the capsid of choice for the muscle-targeting clinical trial that is currently in phase I/II for patients with LOPD (NCT02240407).

However, one limitation of the approach is that muscle targeting via the systemic route requires extremely high doses of vector (exceeding 10^{14} vector genome/kg),[25-27] not easily achievable when addressing the LOPD patient population. In addition, the use of such high vector doses may be unsafe, especially considering the risk of immunotoxicity.[20-24] Actually at these doses, off-target gene transfer in the liver and concomitant inflammation may result in hepatotoxicity.[61] Very recently, in the ASPIRO clinical trial (NCT03199469) for patients suffering from X-linked myotubular myopathy, three out of the seventeen subjects who received the higher AAV vector dose ($3x10^{14}$ vg/kg) showed signs of liver dysfunction, evolving into progressive liver failure.[62] These three young patients died, of sepsis in two patients, and in the third case, the preliminary findings indicated gastrointestinal bleeding. No data on the immune responses are available yet, although it is possible that the chain of events that were observed, and that led to the fatalities in the trial, was to some extent related to inflammation.

This sad event highlight the relevance of the improvement of AAV transduction efficiency in order to reduce the dose that is employed so far in the muscle-targeting trials.[63] To solve this issue, since these last years, efforts are focused in developing alternative serotypes or synthetic capsids presenting better efficiency/specificity for skeletal muscle, heart, and diaphragm.[64-74] Rational design, directed evolution, cell-penetrating peptide are examples of techniques employed to improve muscle-targeting AAV capsids. In addition to capsids optimization, it is also important to develop more potent expression cassettes containing novel promoters that outperform conventional ones[37, 38] and that consequently allow for high and widespread muscle-specific expression at lower and safer vector doses.

The preliminary results reported here underscore the potential of the computational design to improve the performance of a therapeutic vector for muscle-directed gene therapy for myopathies such as PD. In particular, the combination of the *in silico* designed potent muscle-specific CRM/SpC5-12 promoter[41] with the use of innovative muscle-tropic AAV serotypes derived from peptide display and directed molecular evolution technologies, resulted in substantial improvement of muscle-specific transgene expression following systemic gene delivery in GSD II adult mice.

Compared to AAV9, gold standard for muscle-directed gene therapies, the innovative myospecific AAV vectors showed stronger expression levels in all major muscle types. This was consistent with robust correction of the pathological glycogen accumulation in the muscles of GSD II mice. A 10-fold lower dose than the one currently used in several clinical trials with AAV9 (10^{14} vector genome/kg),[25-27] seems to achieve the therapeutic efficacy in GSD II mice. We reported here complete correction of glycogen accumulation in heart, diaphragm and skeletal muscle following systemic administration of $1.6x10^{13}$vg/kg of AAVS1P1-Enh-GAAco.

Importantly, and in contrast to AAV9, we demonstrated that the novel AAV prototypes were markedly detargeted from the liver at the protein and DNA levels. This was the result of the combination of the specific design of these AAV variants and the use of a muscle-tropic promoter, SPc5-12, in our study. This significant liver detargeting may minimize the risk of vector dose-dependent hepatotoxicity.

Although studies in mice provide great opportunities to screen and select interesting novel AAV vectors, further evaluations in NHP models and/or in human cells will be crucial for the success of muscle gene therapy studies, particularly for biological potency and safety assessment.

Human GAA is in general quite immunogenic[75] and as in previous studies in mouse model of PD using muscle-specific promoters,[8, 19, 46] but also in a phase I–II clinical trial using the ubiquitous cytomegalovirus (CMV) promoter[76], we reported the formation of anti-hGAA antibodies following systemic AAV administration. Immunity to GAA is a concern for AAV-mediated gene therapy for PD, because formation of neutralizing antibodies can be associated with a poor prognosis in patients, such as with ERT.[77-79] Research into overcoming immunity through immunomodulation and gene transfer are becoming more and more important to achieve long-term efficacy.

An efficacious approach to induce immune tolerance consists in the elimination of B- and T-cells to prevent antibody production. Immunomodulation with molecules such as rapamycine or tacrolimus, were reported to be effective in preventing infusion-associated reactions during ERT in patients and has shown similar success in the context of gene therapy.[80-84] Novel promising approach to induce immune tolerance employs bioencapsulated delivery of GAA in plant cells[85, 86] or in red blood cells.[87] Further works are still needed in animal models of PD, but it could have an interesting impact in the clinic. However, this approach is restricted to ERT and maybe not adpated to gene therapy. An alternative effective strategy is to target the hGAA expression to the liver, generating antigen-specific regulatory T cells (Tregs).[88-92] In particular, successful prevention of antibody production was achieved when employing a liver-specific promoter to express GAA.[9, 56, 57, 59] However, hepatic expression with AAV vectors has some limits, notably the fact that it cannot persist at long-term in a growing liver.[93] This poses important challenges to the use of this therapeutic strategy in IOPD patients. A solution could come from the use of a dual-AAV vectors approach that targets gene expression to the liver and neuromuscular tissues and may have the potential to be superior in inducing immune tolerance and correction of Pompe disease pathology.

Altogether, the preliminary results obtained in this study highlight very promising myospecific vectors for PD, presenting great potential for augmenting the efficacy and safety of muscle-directed gene therapy, and motivate for further explorations. Developing a clinical product that improves physiologic outcomes and induces tolerance will likely be the most successful intervention for PD.

5. References

1. Mah, C. et al. Sustained correction of glycogen storage disease type II using adeno-associated virus serotype 1 vectors. *Gene Ther* **12**, 1405-9 (2005).
2. Mah, C.S. et al. Gel-mediated delivery of AAV1 vectors corrects ventilatory function in Pompe mice with established disease. *Mol Ther* **18**, 502-10 (2010).
3. Rucker, M. et al. Rescue of enzyme deficiency in embryonic diaphragm in a mouse model of metabolic myopathy: Pompe disease. *Development* **131**, 3007-19 (2004).
4. Elmallah, M.K. et al. Sustained correction of motoneuron histopathology following intramuscular delivery of AAV in pompe mice. *Mol Ther* **22**, 702-12 (2014).
5. Fraites, T.J., Jr. et al. Correction of the enzymatic and functional deficits in a model of Pompe disease using adeno-associated virus vectors. *Mol Ther* **5**, 571-8 (2002).
6. Qiu, K., Falk, D.J., Reier, P.J., Byrne, B.J. & Fuller, D.D. Spinal delivery of AAV vector restores enzyme activity and increases ventilation in Pompe mice. *Mol Ther* **20**, 21-7 (2012).

7. Cresawn, K.O. et al. Impact of humoral immune response on distribution and efficacy of recombinant adeno-associated virus-derived acid alpha-glucosidase in a model of glycogen storage disease type II. *Hum Gene Ther* **16**, 68-80 (2005).
8. Sun, B. et al. Correction of glycogen storage disease type II by an adeno-associated virus vector containing a muscle-specific promoter. *Mol Ther* **11**, 889-98 (2005).
9. Puzzo, F. et al. Rescue of Pompe disease in mice by AAV-mediated liver delivery of secretable acid alpha-glucosidase. *Sci Transl Med* **9** (2017).
10. Sun, B. et al. Efficacy of an adeno-associated virus 8-pseudotyped vector in glycogen storage disease type II. *Mol Ther* **11**, 57-65 (2005).
11. Ziegler, R.J. et al. Ability of adeno-associated virus serotype 8-mediated hepatic expression of acid alpha-glucosidase to correct the biochemical and motor function deficits of presymptomatic and symptomatic Pompe mice. *Hum Gene Ther* **19**, 609-21 (2008).
12. Wang, G. et al. Assessment of toxicity and biodistribution of recombinant AAV8 vector-mediated immunomodulatory gene therapy in mice with Pompe disease. *Mol Ther Methods Clin Dev* **1**, 14018 (2014).
13. Schuster, D.J. et al. Biodistribution of adeno-associated virus serotype 9 (AAV9) vector after intrathecal and intravenous delivery in mouse. *Frontiers in neuroanatomy* **8**, 42 (2014).
14. Falk, D.J. et al. Peripheral nerve and neuromuscular junction pathology in Pompe disease. *Hum Mol Genet* **24**, 625-36 (2015).
15. Todd, A.G. et al. Correcting Neuromuscular Deficits With Gene Therapy in Pompe Disease. *Ann Neurol* **78**, 222-34 (2015).
16. Falk, D.J. et al. Intrapleural administration of AAV9 improves neural and cardiorespiratory function in Pompe disease. *Mol Ther* **21**, 1661-7 (2013).
17. ElMallah, M.K. et al. Retrograde gene delivery to hypoglossal motoneurons using adeno-associated virus serotype 9. *Hum Gene Ther Methods* **23**, 148-56 (2012).
18. Falk, D.J. et al. Comparative impact of AAV and enzyme replacement therapy on respiratory and cardiac function in adult Pompe mice. *Mol Ther Methods Clin Dev* **2**, 15007 (2015).
19. Mah, C. et al. Physiological correction of Pompe disease by systemic delivery of adeno-associated virus serotype 1 vectors. *Mol Ther* **15**, 501-7 (2007).
20. Mingozzi, F. & High, K.A. Immune responses to AAV in clinical trials. *Current gene therapy* **7**, 316-324 (2007).
21. Mingozzi, F. & High, K.A. Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. *Nat Rev Genet* **12**, 341-55 (2011).
22. Mingozzi, F. et al. AAV-1–mediated gene transfer to skeletal muscle in humans results in dose-dependent activation of capsid-specific T cells. *Blood* **114**, 2077-2086 (2009).
23. Pien, G.C. et al. Capsid antigen presentation flags human hepatocytes for destruction after transduction by adeno-associated viral vectors. *The Journal of clinical investigation* **119**, 1688-1695 (2009).
24. Martino, A.T. et al. Engineered AAV vector minimizes in vivo targeting of transduced hepatocytes by capsid-specific CD8+ T cells. *Blood* **121**, 2224-2233 (2013).
25. Childers, M.K. et al. Gene therapy prolongs survival and restores function in murine and canine models of myotubular myopathy. *Science translational medicine* **6**, 220ra10-220ra10 (2014).
26. Le Guiner, C. et al. Long-term microdystrophin gene therapy is effective in a canine model of Duchenne muscular dystrophy. *Nature communications* **8**, 1-15 (2017).
27. Mack, D.L. et al. Systemic AAV8-mediated gene therapy drives whole-body correction of myotubular myopathy in dogs. *Molecular Therapy* **25**, 839-854 (2017).
28. Flotte, T.R. et al. Phase 2 clinical trial of a recombinant adeno-associated viral vector expressing α1-antitrypsin: interim results. *Human gene therapy* **22**, 1239-1247 (2011).
29. Bowles, D.E. et al. Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. *Molecular Therapy* **20**, 443-455 (2012).
30. Buchlis, G. et al. Factor IX expression in skeletal muscle of a severe hemophilia B patient 10 years after AAV-mediated gene transfer. *Blood* **119**, 3038-41 (2012).
31. Mendell, J.R. et al. A phase 1/2a follistatin gene therapy trial for becker muscular dystrophy. *Molecular Therapy* **23**, 192-201 (2015).
32. Carpentier, A.C. et al. Effect of alipogene tiparvovec (AAV1-LPLS447X) on postprandial chylomicron metabolism in lipoprotein lipase-deficient patients. *The Journal of Clinical Endocrinology* **97**, 1635-1644 (2012).
33. Gaudet, D. et al. Efficacy and long-term safety of alipogene tiparvovec (AAV1-LPLS447X) gene therapy for lipoprotein lipase deficiency: an open-label trial. *Gene Ther* **20**, 361-9 (2013).
34. Pacak, C.A., Sakai, Y., Thattaliyath, B.D., Mah, C.S. & Byrne, B.J. Tissue specific promoters improve specificity of AAV9 mediated transgene expression following intra-vascular gene delivery in neonatal mice. *Genetic vaccines and therapy* **6**, 1-5 (2008).

35. Jonuschies, J. et al. The human desmin promoter drives robust gene expression for skeletal muscle stem cell-mediated gene therapy. *Current gene therapy* **14**, 276-288 (2014).
36. Papadakis, E., Nicklin, S., Baker, A. & White, S. Promoters and control elements: designing expression cassettes for gene therapy. *Current gene therapy* **4**, 89-113 (2004).
37. Foster, H. et al. Codon and mRNA sequence optimization of microdystrophin transgenes improves expression and physiological outcome in dystrophic mdx mice following AAV2/8 gene transfer. *Molecular therapy* **16**, 1825-1832 (2008).
38. Li, X., Eastman, E.M., Schwartz, R.J. & Draghia-Akli, R. Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences. *Nature biotechnology* **17**, 241-245 (1999).
39. Somia, N.V., Kafri, T. & Verma, I.M. Piecing together more efficient gene expression. *Nature biotechnology* **17**, 224-225 (1999).
40. Wang, B. et al. Construction and analysis of compact muscle-specific promoters for AAV vectors. *Gene therapy* **15**, 1489-1499 (2008).
41. Sarcar, S. et al. Next-generation muscle-directed gene therapy by in silico vector design. *Nature communications* **10**, 1-16 (2019).
42. Raben, N. et al. Targeted disruption of the acid alpha-glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen storage disease type II. *J Biol Chem* **273**, 19086-92 (1998).
43. Chuah, M.K. et al. Liver-specific transcriptional modules identified by genome-wide in silico analysis enable efficient gene therapy in mice and non-human primates. *Molecular Therapy* **22**, 1605-1613 (2014).
44. Nair, N. et al. Computationally designed liver-specific transcriptional modules and hyperactive factor IX improve hepatic gene therapy. *Blood, The Journal of the American Society of Hematology* **123**, 3195-3199 (2014).
45. Rincon, M.Y. et al. Genome-wide computational analysis reveals cardiomyocyte-specific transcriptional cis-regulatory motifs that enable efficient cardiac gene therapy. *Molecular Therapy* **23**, 43-52 (2015).
46. Sun, B. et al. Correction of multiple striated muscles in murine Pompe disease through adeno-associated virus-mediated gene therapy. *Mol Ther* **16**, 1366-71 (2008).
47. Zhang, P. et al. Immunodominant liver-specific expression suppresses transgene-directed immune responses in murine pompe disease. *Hum Gene Ther* **23**, 460-72 (2012).
48. Ebbink, B.J. et al. Cognitive decline in classic infantile Pompe disease: An underacknowledged challenge. *Neurology* **86**, 1260-1261 (2016).
49. Parini, R. et al. Long term clinical history of an Italian cohort of infantile onset Pompe disease treated with enzyme replacement therapy. *Orphanet journal of rare diseases* **13**, 32 (2018).
50. Prater, S.N. et al. The emerging phenotype of long-term survivors with infantile Pompe disease. *Genetics in medicine* **14**, 800-810 (2012).
51. Schoser, B. et al. Survival and long-term outcomes in late-onset Pompe disease following alglucosidase alfa treatment: a systematic review and meta-analysis. *Journal of neurology* **264**, 621-630 (2017).
52. Van der Ploeg, A.T. et al. Open-label extension study following the Late-Onset Treatment Study (LOTS) of alglucosidase alfa. *Molecular genetics and metabolism* **107**, 456-461 (2012).
53. Park, J.-S., Kim, H.-G., Shin, J.-H., Choi, Y.-C. & Kim, D.-S. Effect of enzyme replacement therapy in late onset Pompe disease: open pilot study of 48 weeks follow-up. *Neurological Sciences* **36**, 599-605 (2015).
54. Papadopoulos, C. et al. Effect of enzyme replacement therapy with alglucosidase alfa (Myozyme®) in 12 patients with advanced late-onset Pompe disease. *Molecular genetics and metabolism* **122**, 80-85 (2017).
55. Orlikowski, D. et al. Recombinant human acid alpha-glucosidase (rhGAA) in adult patients with severe respiratory failure due to Pompe disease. *Neuromuscular Disorders* **21**, 477-482 (2011).
56. Colella, P. et al. AAV Gene Transfer with Tandem Promoter Design Prevents Anti-transgene Immunity and Provides Persistent Efficacy in Neonate Pompe Mice. *Mol Ther Methods Clin Dev* **12**, 85-101 (2019).
57. Cagin, U. et al. Rescue of advanced Pompe disease in mice with hepatic expression of secretable acid α-glucosidase. *Molecular Therapy* (2020).
58. Ronzitti, G., Collaud, F., Laforet, P. & Mingozzi, F. Progress and challenges of gene therapy for Pompe disease. *Ann Transl Med* **7**, 287 (2019).
59. Han, S.O. et al. Low-Dose Liver-Targeted Gene Therapy for Pompe Disease Enhances Therapeutic Efficacy of ERT via Immune Tolerance Induction. *Mol Ther Methods Clin Dev* **4**, 126-136 (2017).
60. Schuster, D.J. et al. Biodistribution of adeno-associated virus serotype 9 (AAV9) vector after intrathecal and intravenous delivery in mouse. *Front Neuroanat* **8**, 42 (2014).
61. Mendell, J.R. et al. Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. *N Engl J Med* **377**, 1713-1722 (2017).

62. Audentes. (https://www.joshuafrase.org/get-involved/recensus-study.php, 2020).
63. Wilson, J.M. & Flotte, T.R. Moving Forward after Two Deaths in a Gene Therapy Trial of Myotubular Myopathy: To learn from these tragedies, the scientific community must commit to full transparency and cooperation. *Genetic Engineering & Biotechnology News* **40**, 14, 16 (2020).
64. Bello, A. et al. Novel adeno-associated viruses derived from pig tissues transduce most major organs in mice. *Scientific reports* **4**, 6644 (2014).
65. Tulalamba, W. et al. Distinct transduction of muscle tissue in mice after systemic delivery of AAVpo1 vectors. *Gene Therapy* **27**, 170-179 (2020).
66. Choudhury, S.R. et al. In vivo selection yields AAV-B1 capsid for central nervous system and muscle gene therapy. *Molecular Therapy* **24**, 1247-1257 (2016).
67. Yang, L. et al. A myocardium tropic adeno-associated virus (AAV) evolved by DNA shuffling and in vivo selection. *Proceedings of the National Academy of Sciences* **106**, 3946-3951 (2009).
68. Yang, L., Li, J. & Xiao, X. in Muscle Gene Therapy 127-139 (Springer, 2011).
69. Li, C. et al. Development of patient-specific AAV vectors after neutralizing antibody selection for enhanced muscle gene transfer. *Molecular Therapy* **24**, 53-65 (2016).
70. Nance, M.E. & Duan, D. in Muscle Gene Therapy 193-206 (Springer, 2019).
71. Yu, C.-Y. et al. A muscle-targeting peptide displayed on AAV2 improves muscle tropism on systemic delivery. *Gene therapy* **16**, 953-962 (2009).
72. Work, L.M. et al. Development of efficient viral vectors selective for vascular smooth muscle cells. *Molecular therapy* **9**, 198-208 (2004).
73. Ying, Y. et al. Heart-targeted adeno-associated viral vectors selected by in vivo biopanning of a random viral display peptide library. *Gene therapy* **17**, 980-990 (2010).
74. Nance, M.E. & Duan, D. Perspective on adeno-associated virus capsid modification for Duchenne muscular dystrophy gene therapy. *Human gene therapy* **26**, 786-800 (2015).
75. Brooks, D.A., Kakavanos, R. & Hopwood, J.J. Significance of immune response to enzyme-replacement therapy for patients with a lysosomal storage disorder. *Trends in molecular medicine* **9**, 450-453 (2003).
76. Corti, M. et al. Safety of Intradiaphragmatic Delivery of Adeno-Associated Virus-Mediated Alpha-Glucosidase (rAAV1-CMV-hGAA) Gene Therapy in Children Affected by Pompe Disease. *Hum Gene Ther Clin Dev* **28**, 208-218 (2017).
77. Kishnani, P.S. et al. Cross-reactive immunologic material status affects treatment outcomes in Pompe disease infants. *Mol Genet Metab* **99**, 26-33 (2010).
78. Kazi, Z.B. et al. Sustained immune tolerance induction in enzyme replacement therapy-treated CRIM-negative patients with infantile Pompe disease. *JCI Insight* **2** (2017).
79. Poelman, E. et al. High sustained antibody titers in patients with classic infantile Pompe disease following immunomodulation at start of enzyme replacement therapy. *The Journal of pediatrics* **195**, 236-243. e3 (2018).
80. Moghimi, B. et al. Induction of tolerance to factor VIII by transient co-administration with rapamycin. *Journal of Thrombosis and Haemostasis* **9**, 1524-1533 (2011).
81. Battaglia, M., Stabilini, A. & Roncarolo, M.-G. Rapamycin selectively expands CD4+ CD25+ FoxP3+ regulatory T cells. *Blood* **105**, 4743-4748 (2005).
82. Elder, M.E. et al. B-Cell depletion and immunomodulation before initiation of enzyme replacement therapy blocks the immune response to acid alpha-glucosidase in infantile-onset Pompe disease. *The Journal of pediatrics* **163**, 847-854. e1 (2013).
83. Corti, M. et al. B-cell depletion is protective against anti-AAV capsid immune response: a human subject case study. *Molecular Therapy-Methods & Clinical Development* **1**, 14033 (2014).
84. Ishii, A. et al. rAAV8 and rAAV9-mediated long-term muscle transduction with tacrolimus (FK506) in non-human primates. *Molecular Therapy-Methods & Clinical Development* (2020).
85. Kwon, K.-C. & Daniell, H. Oral delivery of protein drugs bioencapsulated in plant cells. *Molecular Therapy* **24**, 1342-1350 (2016).
86. Wang, X. et al. Plant-based oral tolerance to hemophilia therapy employs a complex immune regulatory response including LAP+ CD4+ T cells. *Blood* **125**, 2418-2427 (2015).
87. Cremel, M. et al. Innovative approach in Pompe disease therapy: induction of immune tolerance by antigen-encapsulated red blood cells. *International journal of pharmaceutics* **491**, 69-77 (2015).
88. Cao, O. et al. Induction and role of regulatory CD4+CD25+ T cells in tolerance to the transgene product following hepatic in vivo gene transfer. *Blood* **110**, 1132-40 (2007).
89. Mingozzi, F. et al. Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer. *J Clin Invest* **111**, 1347-56 (2003).
90. Dobrzynski, E. et al. Induction of antigen-specific CD4+ T-cell anergy and deletion by in vivo viral gene transfer. *Blood* **104**, 969-77 (2004).
91. Mingozzi, F. et al. Modulation of tolerance to the transgene product in a nonhuman primate model of AAV-mediated gene transfer to liver. *Blood* **110**, 2334-2341 (2007).

92. Keeler, G.D., Markusic, D.M. & Hoffman, B.E. Liver induced transgene tolerance with AAV vectors. *Cellular immunology* **342**, 103728 (2019).
93. Bortolussi, G. et al. Life-long correction of hyperbilirubinemia with a neonatal liver-specific AAV-mediated gene transfer in a lethal mouse model of Crigler-Najjar Syndrome. *Hum Gene Ther* **25**, 844-55 (2014).

Chapter 5

Pharmacological chaperones to enhance investigational gene therapy for Pompe disease

This work is part of a manuscript in revision
Costa-Verdera H, Collaud F, Riling C.R et al, (2021)
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1. Introduction

Gene therapy (GT) approaches have been investigated to treat Pompe disease (PD). For example, we previously demonstrated that the use of a nucleic acid molecule encoding a truncated acid alpha-glucosidase (GAA) polypeptide fused with a signal peptide improves the tissue uptake of GAA.[1, 2] In particular, at the 24th annual congress of the World Muscle Society (WMS), in October 2019, Spark therapeutics presented a progress report on this GT project and a summary of the preclinical results: the administration of the drug candidate called SPK-3006, increases the lifespan, reduces glycogen accumulation and improves the cardiac, respiratory and muscular functions of mouse model of PD, compared to untreated mice. Results on muscle strength were better with this GT than with enzyme replacement therapy at 20mg/kg.[3] In non-human primates, a single infusion of SPK-3006 results in dose-dependent secretion of the enzyme GAA, which increases circulating GAA levels. Supported by these solid results, the translation to the clinic is expected soon, with a first inclusion waited for the end of 2020. However, it is important to note that despite encouraging improvements in Pompe mice with this treatment, glycogen accumulation was only partially rescued in the central nervous system (CNS).

Therefore, there is still a need to provide better therapies for treating PD. In particular, there is a need to provide better therapies for increasing the uptake of GAA in a tissue of the nervous system, for treating CNS dysfunctions in GSD, for improving the respiratory neuromuscular function and/or for decreasing respiratory impairments in a subject having a GSD.

On this work, we decided to investigate the potential of the combination of small α-glucosidase inhibitors as pharmacological chaperones (PCs) with AAV GT, to enhance the stability of GAA and increase the GAA tissue uptake. Among several potential lead compounds,[4, 5] we investigated the action of PCs already described as promoting GAA activity, such as 1-deoxynojirimycin (DNJ, AT2220, duvoglustat)[6-9], and a combination of ambroxol (ABX) and DNJ.[10]

Overall, our data suggest that the combination of PCs, especially DNJ-ABX association, has a synergic effect, when combined with GT and resulted in improved enzyme activity in tissues and reduction of glycogen accumulation in mice. We have shown beneficial effects, such as enhancement of circulating GAA levels and uptake in tissues, which could help to reduce AAV vector doses, minimizing potential dose-related toxicity. Additional investigations will allow to confirm these encouraging results and will help to define the therapeutic efficacy required to improve correction of the pathogenic accumulation of glycogen in tissues of the nervous system.

2. Materials and methods

2.1 Test Items

GAA expression cassette and AAV vectors

A codon-optimized human GAA (hGAA) coding sequences was cloned in an AAV vector backbone containing the wild-type AAV2 ITRs, the apolipoprotein E hepatocyte control region enhancer, the human alpha 1-antitrypsin (hAAT) promoter and a bovine growth hormone (bGH) polyA signal. The native GAA signal peptide (amino acids 1-27, starting from the ATG) was removed and replaced by the one of chymotrypsinogen B2 (sp7) and

amino acids 28-35 of the GAA pro-peptide were removed to generate the sp7-Δ8 version of the transgene, as previously described.[1]

AAV vectors used in this study were produced using an adenovirus-free transient transfection method as described earlier.[11] Titers of AAV vector stocks were determined using quantitative real-time PCR (qPCR) and confirmed by SDS-PAGE followed by SYPRO Ruby protein gel stain and band densitometry. All vector preparations used in the study were quantified side-by-side at least 3 times before use.

Pharmacological chaperones

Commercially available molecules described as competitive inhibitors of GAA or allosteric chaperones that could increase the stability of recombinant GAA, were selected for this study : Duvoglustat-HCl (later referred as DNJ; 100mg/kg per day; Interchim, SanDiego, CA), and Ambroxol-HCl (later referred as ABX; 25mg/kg per day: Sigma-Aldrich, Saint-Louis, MO).

2.2 *In vivo* studies

Mice

Mouse studies were performed according to the French and European legislation on animal care and experimentation (2010/63/EU) and approved by the local Institutional Ethical Board (protocols no. 2015-008 and 2017-011B, apafis no. 13643). Mice were housed in ventilated cages with PLus-10 bedding (SAFE, Augy, France) and kept in a temperature-controlled environment with 12/12-hour light–dark cycle. The SPF status of the animals was maintained throughout the study.

The wild-type (wt) C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The *Gaa*$^{-/-}$ *Cd4*$^{-/-}$ mice used in this study were resulting from the crossing of the mouse strains B6;129-Gaa^{tm1Rabn}/J (stock no 004154, 6neo; Jackson Laboratory, Bar Harbor, ME) originally generated by Raben et al.[12] and B6.129S2-Cd4^{tm1Mak}/J (stock no. 002663; Jackson Laboratory, Bar Harbor, ME). The *Gaa*$^{+/+}$ mice are derived from the same colony and breed separately. Genotypes were confirmed by polymerase chain reaction (PCR) performed on genomic DNA with oligonucleotides specific for the mutated regions in each gene.

Gaa (mutant)	forward 5’-CGTTGGCTACCCGTGATATT-3’
Gaa (wild-type)	forward 5’-TCCTGAGCCCAAACACTTCT-3’
Gaa	reverse 5’-ATTGTTGCACAACGCTCTTG-3’
Cd4 (mutant)	forward 5’-GTGTTGGGTCGTTTGTTCG-3’
Cd4 (wild-type)	forward 5’-CCTCTTGGTTAATGGGGGAT-3’
Cd4	reverse 5’-TTTTTCTGGTCCAGGGTCAC-3’

Diet and Water

Mice received a standard chow diet (certified irradiation sterilized dry granule diet, SAFE, Augy, France) and filter (0.22 μm) sterilized water provided ad libitum.

For the pharmacological treatment, chaperone molecules were diluted in filter sterilized water and provided ad libitum using a "3 on/4 off" regimen, as previously described.[7] This regimen consisted in three consecutive days of treatment followed by four consecutive days with drinking water only.

Gene transfer procedures

Pilot study in wild-type mice

Six to eight-week-old C57Bl/6 male mice were treated intravenously by $5x10^{11}$ vector genome (vg)/kg of an AAV8 vector expressing secretable human GAA (AAV-GAA), via tail vein injection (n=5 animals per group). Two months later, mice were orally treated for four weeks with vehicle (water) or one of the seven pharmacological chaperones (PCs) or combination of them, dissolved in the drinking water, using a "3 days on/4 days off" regimen. Untreated littermates were used as control. Blood sampling, monitoring of body weight and drinking water consumption were performed regularly all along the procedure. At the end of the study (3 months after AAV injection), all mice were sacrificed to harvest tissues. Liver, heart, brain, spinal cord, diaphragm, quadriceps, and triceps were snap-frozen in liquid nitrogen and stored at -80°C until processing.

Combination therapy with ERT

Three to four-month-old GAA deficient male mice were treated intravenously by ERT (alglucosidase alpha, 20mg/kg) in combination with PCs dissolved in the drinking water. Fifteen minutes before ERT infusion, mice were treated with 25 mg/kg of diphenydramine hydrochloride (anti-histaminic) to avoid anaphylaxis. Untreated GAA wild-type mice, untreated GAA deficient mice and GAA deficient mice treated by ERT alone were used as controls. Blood sampling was performed three hours post ERT.

Efficacy studies in GSD II mice

Three to four-month-old GAA deficient male mice received either vehicle (water) or one of the selected PCs dissolved in the drinking water, following a "3 days on/4 days off" regimen for 8 weeks (n=8 animals per group). One day later, vehicle (PBS) or AAV-GAA ($1x10^{11}$vg/kg, minimal effective dose) were administered via tail vein injection. Blood samples, monitoring of body weight and drinking water consumption were performed regularly all along the procedure. Muscle strength was evaluated at the end of the study (two months after initial treatment) and then mice were bled and sacrificed to harvest tissues (at the end of the last washout period). Liver, heart, brain, spinal cord, diaphragm, quadriceps, and triceps were snap-frozen in liquid nitrogen and stored at -80°C until processing to evaluate the biochemical correction.

An additional long-term study was performed. Three to four-month-old GAA deficient male mice were intravenously injected with vehicle (PBS) or AAV-GAA at $6x10^{11}$vg/kg (n=8 animals per group). One month later, mice were orally treated with vehicle (water) or a combination of PCs dissolved in drinking water, following a "3 days on/4 days off" regimen during four months. At the end of the study (five months after AAV injection), mice were bled and sacrificed to harvest tissues. Tissues were snap-frozen in liquid nitrogen and stored at -80°C until processing to evaluate the biochemical correction.

Glycaemia measurement

Blood samples were collected by retro-orbital puncture. At each blood sampling, one drop was used to determine glycaemia by the Accu-Chek Performa system (Roche, Basel, Switzerland), following the manufacturer instructions.

GAA activity assay

Snap-frozen tissues were homogenized in UltraPure DNase- and RNase-free distilled water (Thermo Fisher Scientific). Tissues were weighed, homogenized, and centrifuged for 10 min at 10,000×g to collect the supernatant. The enzymatic reaction was set up using 10µL of tissue homogenate or plasma samples, diluted appropriately and 20µL of substrate, 4-methylumbelliferone (4MU)α-D-glucoside, in black 96-well plates (PerkinElmer). The reaction mixture was incubated at 37°C for 1hr and then stopped by adding 150µL of sodium carbonate buffer (pH 10.5). A standard curve (0–2,500pmol/µL of 4MU) was used to measure released fluorescent 4MU from the individual reaction mixture using the EnSpire Alpha plate reader (PerkinElmer) at 449 nm (emission) and 360 nm (excitation). The protein concentration of the clarified supernatant was quantified by BCA (Thermo Fisher Scientific). To calculate the GAA activity in tissues, the released 4MU concentration was divided by the sample protein concentration, and activity was reported as nmol/hr/mg protein.

Western blot analysis

Total proteins concentration of tissue homogenates, prepared as described above, was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) following manufacturer instructions and protein extracts were separated on a 4-12% bis-tris polyacrylamide gel (Thermo Fisher Scientific, Waltham, MA). The same amount of protein was loaded in each lane. The gel was transferred onto a nitrocellulose membrane and blotted with anti-GAA antibody (AbcamCambridge, UK). Anti-GAPDH antibody (Thermo Fisher Scientific, Waltham, MA) was used as loading control. Secondary antibodies and detection system were from Li-Cor Biosciences (Lincoln, NE).

Glycogen content measurement

Glycogen content was analyzed in tissue homogenates, prepared as previously described. 20µl of homogenate properly diluted was mixed with 55µl of distilled water, incubated for 5min at 95°C, and cooled at 4°C. Next, glycogen contained in samples was digested with amyloglucosidase from Aspergillus Niger (Sigma Aldrich, Saint-Louis, MO), in order to release the glucose monomers. For that, 25µl of amyloglucosidase diluted 1:50 in 0.1M potassium acetate pH5.5 was added to each sample. A control reaction without amyloglucosidase was done in parallel for each sample. Both digestion and control samples were incubated at 37°C for 90min, followed by 5min at 95°C. Glycogen content was determined indirectly by quantifying the number of glucose molecules released per gram of protein, with the use of a colorimetric glucose assay kit (Sigma Aldrich, Saint-Louis, MO). Glucose assay was performed according to the manufacturer's instructions. Absorbance was measured with an EnSpire alpha plate reader (PerkinElmer) at 540 nm**.**

Functional Assessment

Muscle grip strength was measured, as already reported,[13] by the TREAT-NMD Network (https://treat-nmd.org). With the use of a grip-strength meter (Columbus Instruments, San Diego, CA), three independent measurements of the four limbs' strength were calculated. Mean values of the grip strength were reported.

Forelimbs wire-hanging test was performed as already reported.[13] A 4-mm wire was used to record the number of fall over a period of 3 minutes. The number of falls per minute was reported.

Statistical analysis

Results are expressed as mean ± SD as described in the text. The Prism package (Graph Pad Software, La Jolla, CA) was used to analyze data. Statistical analysis has been performed by one or two way ANOVA as described in the text. Values of $p< 0.05$ were considered as statistically significant.

3. Results

3.1 PCs increase GAA delivery and uptake in wild-type mice

Evaluation of the combined effect of PCs and GT was performed in adult C57BL/6 male mice. Mice were injected with a low dose of an AAV vector expressing secretable hGAA under the control of a liver-specific promoter (AAV8-sp7-Δ8-coGAA; $5x10^{11}$ vg/kg). PCs (or water as vehicle) were administered in the drinking water of the mice, 2 months after AAV injection (**Figure 5.1A**). DNJ and DNJ-ABX were well tolerated by the mice, as shown by no significant changes in weight or glycemia (**Figure 5.1B-C**). Drinking water consumption was similar in all groups with a mean volume of around 5.5mL per mouse per day (data not shown).

After one month of PCs treatment, an increase of hGAA activity was observed in blood of DNJ- and DNJ-ABX-treated mice compared to those receiving only water (**Figure 5.1D**), although this difference did not achieve statistical significance. We confirmed these data with the analysis of hGAA levels in blood by Western blot with anti-hGAA antibody. At sacrifice, greater circulating hGAA protein levels were measured in blood of mice treated with PCs compared to mice drinking only water (**Figure 5.1E**). Presence of viral genome was detected in liver of all AAV-treated groups with similar number of copies (**Figure 5.1F**), suggesting an equivalent liver transduction efficacy and a similar transgene expression.

Next, we measured the hGAA levels in muscular and nervous tissues. The 110kDa-immature form of hGAA secreted by the liver is taken up by the tissues. The 70kDa-mature form, is obtained only after proteolytic digestion of the precursor in lysosomes. Therefore, this form is intracellular and its quantification allows estimating the uptake in tissues. Here, despite some heterogeneity among mice, the quantification of the mature lysosomal form of hGAA in tissues by western blot, showed increased levels of the enzyme in some muscles, especially in triceps, and diaphragm of mice treated with PCs (**Figure 5.2A**), while smaller differences were observed in heart and quadriceps. Interestingly the combination of DNJ and ABX led to a significant increase in the levels of hGAA in spinal cord compared to levels

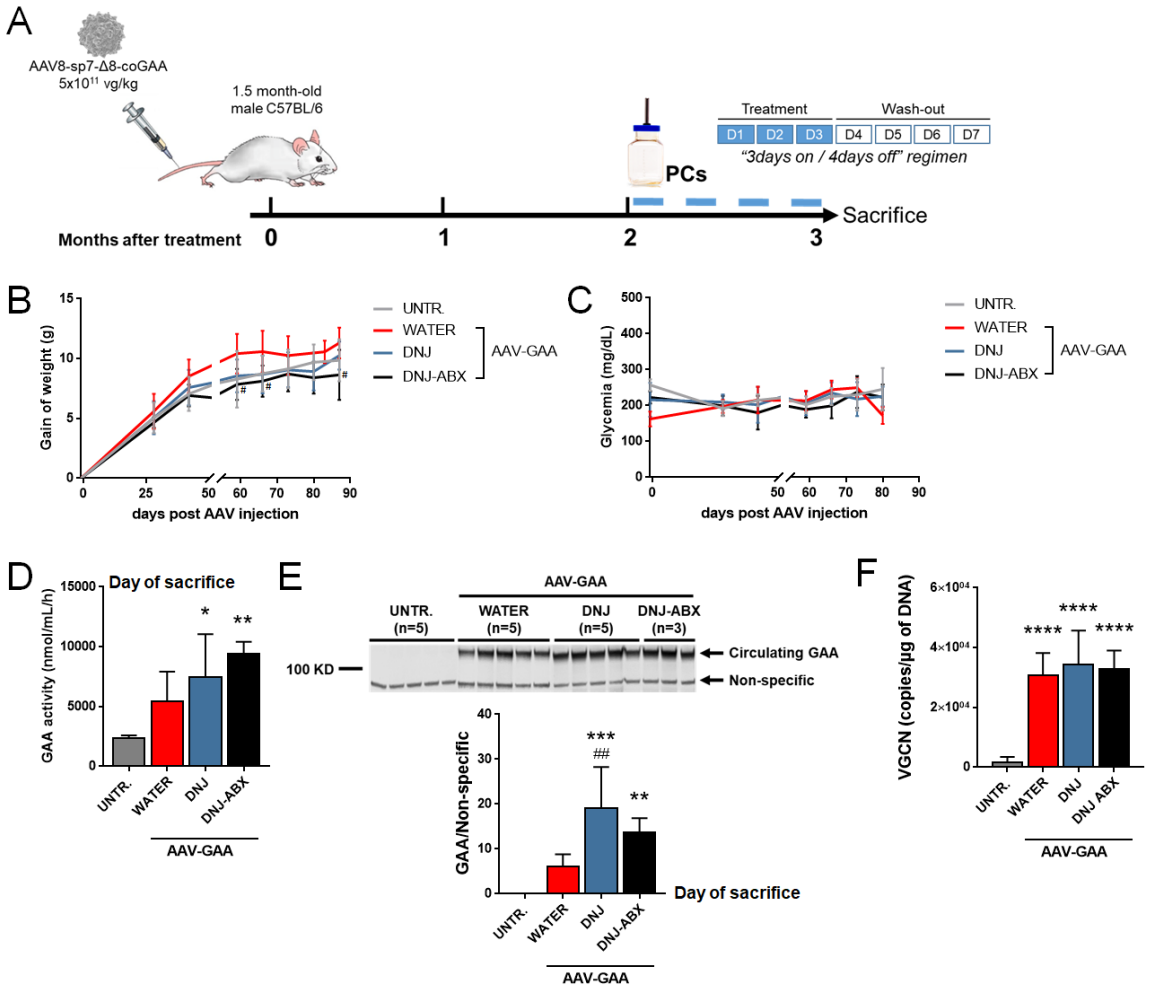


Figure 5.1 PCs increase activity and levels of circulating hGAA in wild-type mice
(A) Scheme of the experimental design. 6-8-week-old C57Bl/6J male mice were injected intravenously at day0 with an AAV8-sp7-Δ8-coGAA (AAV-GAA) vector at 5x10[11] vg/kg. 2 months later, pharmacological chaperones (PCs) were orally administered in mice for four weeks, following a "3 days on / 4 days off" regimen. (n=5/group). Body weight (B) and glycemia (C) of all mice were monitored during 3 months. (D) Measurement of GAA activity levels in blood at sacrifice (day 85). (E) Western blot comparison of circulating GAA protein levels in wild-type mice at sacrifice (day 85-87). Quantification of circulating GAA protein bands normalized by the non-specific band is plotted on the bottom. (F) Vector genome copy number (VGCN) per µg of DNA measured by qPCR in liver of injected mice at day 85. All data are shown as mean ± SD. Statistical analysis : (B) Two-way ANOVA with Tukey post-hoc test; (D, E and F) One-way ANOVA with Tukey post-hoc test; (and # p<0.05, ** and ## p<0.01, ### p<0.001, **** and #### p<0.0001; n = 5 mice per group; except for the group AAV-GAA DNJ-ABX, n=3).*

measured in mice drinking DNJ alone or water (**Figure 5.2B**). No differences among the different AAV-treated cohorts were observed in brain.

Together, these data support a positive effect of PCs on circulating levels of hGAA and uptake in tissues. Interestingly, a synergic effect was observed after administration of DNJ and ABX in GT-treated animals.

3.2 PCs ameliorate circulating GAA levels and uptake in tissues and enhance glycogen clearance in Pompe mice

Based on these encouraging results obtained in wild-type mice with PCT, we selected the combination DNJ-ABX to continue investigations in a relevant animal model of PD. We used a double knock-out *Gaa*$^{-/-}$ *Cd4*$^{-/-}$ mouse model, generated in our lab to overcome the

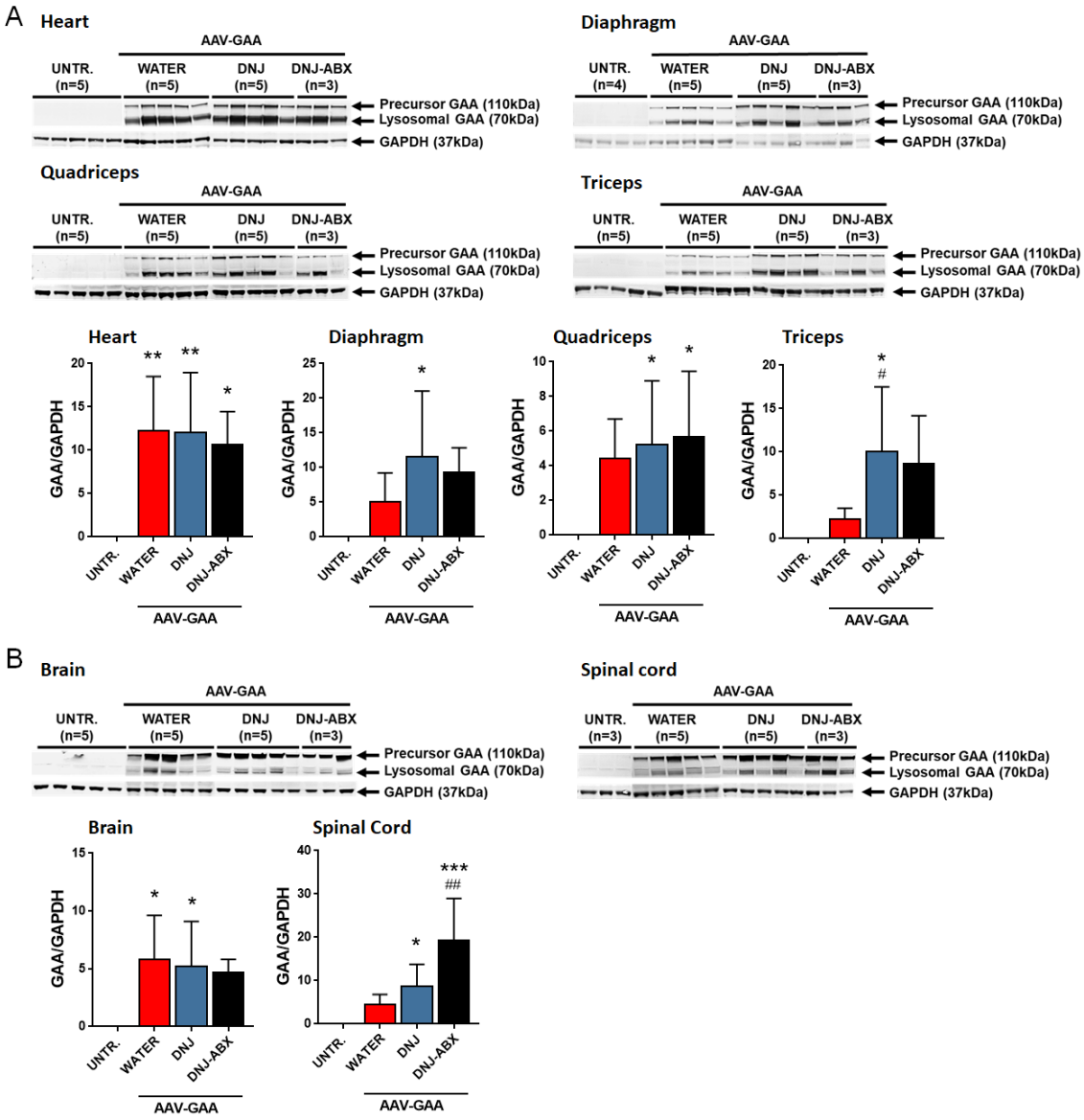


Figure 5.2 PCs increase hGAA delivery and uptake in wild-type mice
Western blot comparison of GAA protein levels in (A) muscles and (B) CNS tissues of wild-type mice, 1 month after chaperone therapy (day 85-87). Quantification of lysosomal GAA band is plotted on the bottom. An anti-GAPDH antibody was used as loading control. Error bars represent SD of the mean. Asterisks (*) indicate significant differences compared to untreated mice (UNTR.), hash symbols (#) indicate significant differences compared to AAV-GAA treated mice (WATER) Statistical analysis was performed by One-way ANOVA with Tukey post-hoc test (and # p<0.05, ** and ## p<0.01, ### p<0.001, **** and #### p<0.0001; n = 5 mice per group; except for the group AAV-GAA DNJ-ABX, n=3).*

detrimental and often lethal anti-rhGAA immune responses generated in GAA deficient mice upon ERT infusions.

Effects of DNJ-ABX used as PCs for GSD II were never evaluated *in vivo*, so we first confirmed the synergic effect of the combined administration of DNJ and ABX to enhance rhGAA bioavailability in ERT-treated mice at a clinically relevant dose (20mg/kg). Because of a relatively short half-life of rhGAA, measurement of circulating levels and activity were done at 3 hours post ERT infusion (**Figure 5.3A**). We observed an increase of circulating rhGAA levels and activity in mice co-treated with the combination ERT and DNJ-ABX (**Figure 5.3B-C**).

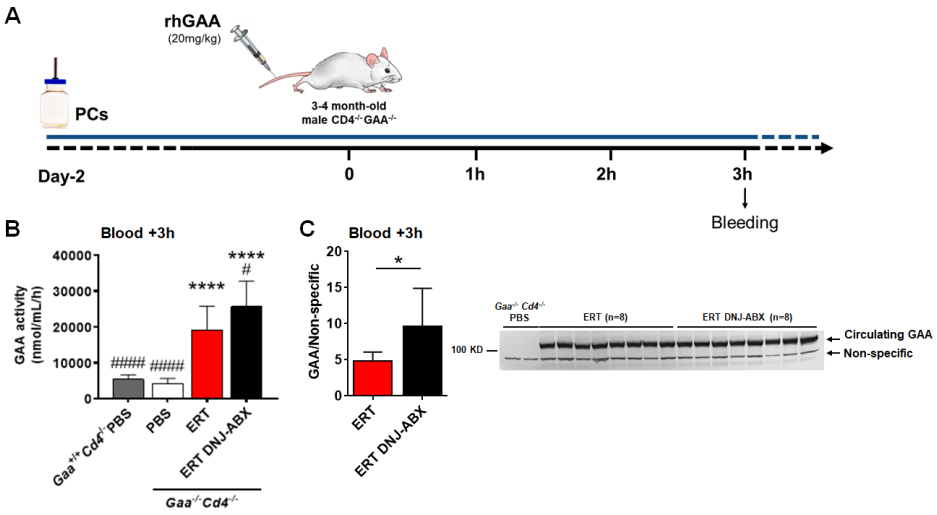


Figure 5.3 PCs improve rhGAA bioavailability in Pompe mice
(A) Scheme of the experimental design. 3 to 4-month-old immunocompromised $Gaa^{-/-}Cd4^{-/-}$ male mice were intravenously injected with rhGAA (ERT) at a dose of 20 mg/kg together with an intraperitoneal injection of an antihistaminic drug (diphenhydramine hydrochloride, 25mg/kg) to further prevent the risk of anaphylaxis. A regimen of DNJ and ABX, was dissolved in the drinking water and orally administered to one of the cohorts (n = 8 per cohort). PBS-injected $Gaa^{+/+}Cd4^{-/-}$ and $Gaa^{-/-}Cd4^{-/-}$ mice were used as controls. 3 hours after ERT, blood samples were collected for the analysis of circulating GAA activity and levels. (B) Measurement of rhGAA activity levels in plasma. Asterisks (*) indicate significant differences compared to Gaa+/+Cd4-/- PBS; hash symbols (#) indicate significant differences compared to $Gaa^{-/-}Cd4^{-/-}$ ERT. (C) Western blot comparison of circulating rhGAA protein levels in $Gaa^{-/-}Cd4^{-/-}$ mice treated with ERT with or without DNJ-ABX. Quantification of circulating GAA protein bands normalized by the non-specific band is plotted on the right. All data are shown as mean ± SD. Statistical analysis: (B) One-way ANOVA with Tukey post-hoc test; (C) t-test (and # p<0.05, ** and ## p<0.01, ### p<0.001, **** and #### p<0.0001, n = 8 mice per group).*

Next, in order to inquire the effects of these PCs following this protocol on GT efficacy, another study was performed in AAV-treated *$Gaa^{-/-}Cd4^{-/-}$* mice, where PCs (or water as vehicle) were orally administered for two months (**Figure 5.4A**). Blood analyses over time showed an increase in circulating GAA activity in PCs-treated animals compared to mice treated by AAV GT alone (**Figure 5.4B**). We confirmed these data with the analysis of hGAA levels in blood by Western blot. At sacrifice (day 66), circulating hGAA was detectable in all AAV-treated *$Gaa^{-/-}Cd4^{-/-}$* mice with a significant increase observed in PCs-treated animals (**Figure 5.4C**). These results were also associated with an increase of GAA expression in the liver (**Figure 5.4D**) and we also noted a slight increase of vector genome copy number (VGCN) measured by qPCR in the liver at sacrifice in mice treated by PCT (**Figure 5.4E**). Even if this increase of VGCN was moderate and with values obtained in the same order of magnitude, these results were unexpected since all mice were injected at the same dose and with the same AAV batch. To evaluate the effect of DNJ-ABX on liver tranduction, we performed another experiment, in which DNJ-ABX drinking solution was administered to adult mice 3 days before, and 4 days or 11 days after a systemic injection of an AAV8 expressing human factor IX (hFIX) (**Figure 5.5A**). Similar levels of transduction and hFIX expression were observed in all treatment groups with no effect of DNJ-ABX on efficacy (**Figure 5.5B**).

In line with the increased circulating hGAA availability and liver transgene expression, we observed in PCT-treated GSD II mice an increased delivery of hGAA in muscle, leading to a better correction of the disease phenotype. At the end of the study (at day 66), heart muscle rescue was observed only in DNJ-ABX treated group with the normalization of the heart to body weight ratio **(Figure 5.6A**). Muscle function was already improved two months after treatment in AAV-treated cohorts compared to control *$Gaa^{-/-}Cd4^{-/-}$* animals, independently of the PCT (**Figure 5.6B-C**).

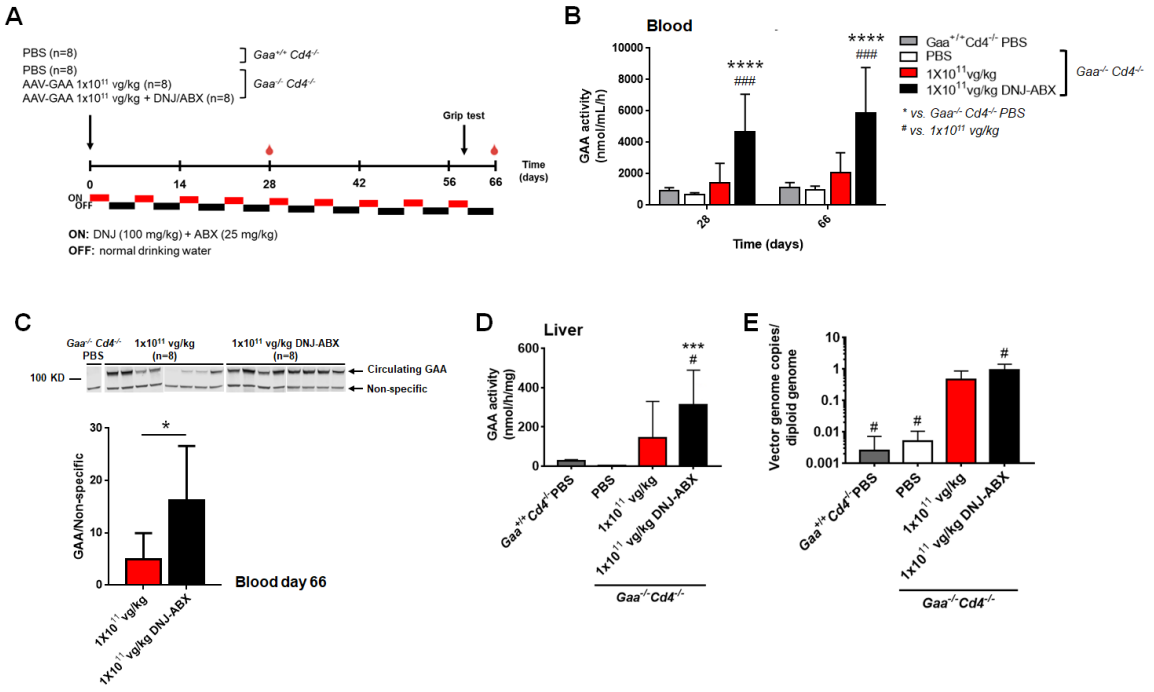


Figure 5.4 PCs increase activity and levels of circulating hGAA in Pompe mice
(A) Scheme of the experimental design. 3-4 month-old Gaa$^{-/-}$Cd4$^{-/-}$ mice received a single injection of AAV-GAA at 1x10^{11}vg/kg with or without pharmacological chaperones (DNJ-ABX) dissolved in the drinking water, orally administered to the mice during 2 months, using a "3 days on/4 days off" regimen. (n = 8 per cohort). PBS-injected Gaa$^{+/+}$Cd4$^{-/-}$ and Gaa$^{-/-}$Cd4$^{-/-}$ mice were used as controls. Blood samples were collected every months for the analysis of circulating GAA activity. Mice were sacrificed after 2 months of follow-up, at day 66. (B) Measurement of GAA activity levels in plasma at the indicated time-points. Asterisks (*) indicate significant differences compared to Gaa$^{-/-}$Cd4$^{-/-}$ PBS; hash symbols (#) indicate significant differences compared to Gaa$^{-/-}$Cd4$^{-/-}$ AAV-GAA 1x10^{11}vg/kg. (C) Western blot comparison of circulating GAA protein levels in Gaa$^{-/-}$Cd4$^{-/-}$ mice treated with 1x10^{11}vg/kg AAV-GAA with or without DNJ-ABX at 2 months. Quantification of circulating GAA protein bands normalized by the non-specific band is plotted on the bottom. (C) (D) Analysis of vector genome copies per diploid genome in liver at sacrifice (day 66), determined by qPCR. Hash symbols (#) indicate significant differences compared to Gaa$^{-/-}$Cd4$^{-/-}$ AAV-GAA 1x10^{11}vg/kg. (E) Analysis of GAA uptake in liver at sacrifice. Asterisks (*) indicate significant differences compared to Gaa$^{-/-}$Cd4$^{-/-}$ PBS, hash symbols (#) indicate significant differences compared to Gaa$^{-/-}$Cd4$^{-/-}$ AAV-GAA 1x10^{11}vg/kg. All data are shown as mean ± SD. Statistical analysis: (B) Two-way ANOVA with Tukey post-hoc test; (C) t-test; (D-E) One-way ANOVA with Tukey post-hoc test; (and # p<0.05, ** and ## p<0.01, ### p<0.001, **** and #### p<0.0001, n = 8 mice per group).*

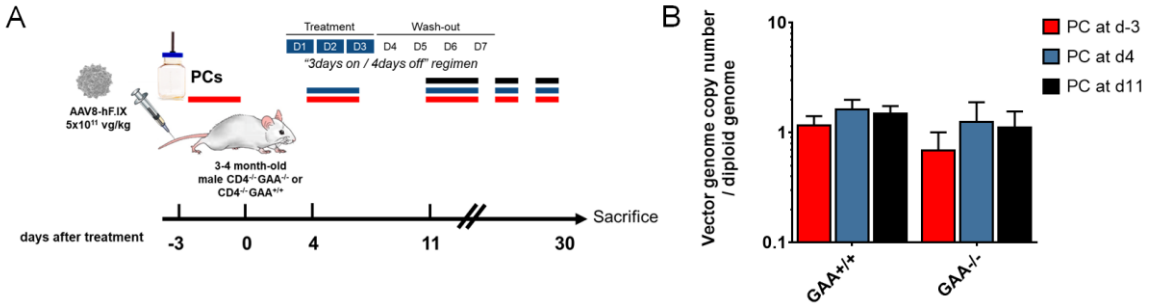


Figure 5.5 PCs administration before or after GT do not impact VGCN values.
(A) Scheme of the experimental design. Three to four-month-old Cd4$^{-/-}$ Gaa$^{-/-}$ male mice or Cd4$^{-/-}$ Gaa$^{+/+}$ male mice were intravenously injected with an AAV8 vector expressing the human coagulation factor IX (AAV8-hF.IX) at 5x10^{11} vg/kg in combination with pharmacological chaperones (PCs) dissolved in the drinking water, orally administered to the mice during 1 month, using a "3 days on/4 days off" regimen. This regimen was composed by DNJ and ABX molecules and started 3 days before, 4 or 11 days after gene therapy (n=8/group). At the end of the study (day 30), mice were bled and sacrificed for liver collection (B) Vector genome copy number (VGCN) per cell was measured by qPCR in liver of injected mice. Data are shown as mean ± SD. Statistical analysis: One-way ANOVA with Tukey post-hoc test.

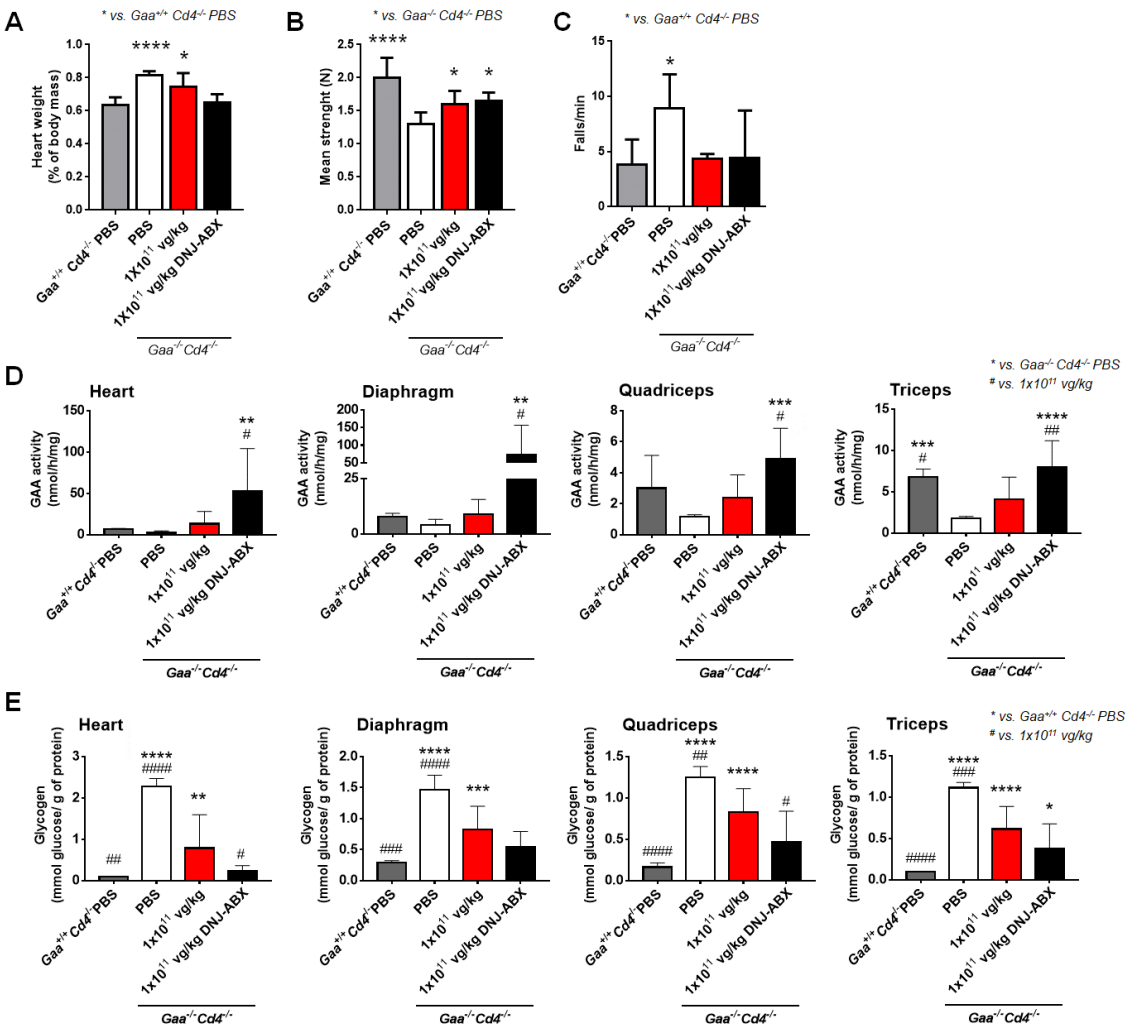


Figure 5.6 PCT enhances the gene therapy efficacy in Pompe mice
GSD II mice were treated as described in Figure 5.4 (n=8/group). (A-C) Evaluation of cardiac and skeletal muscle function. (A) Evaluation of cardiac hypertrophy expressed as percentage of body weight. Asterisks (*) indicate significant differences compared to $Gaa^{+/+}Cd4^{-/-}$ PBS. (B) Measurement of grip strength after 2 months of treatment. Asterisks (*) indicate significant differences compared to $Gaa^{-/-}Cd4^{-/-}$ PBS. (C) Wire hang evaluation after 2 months of treatment. Asterisks (*) indicate significant differences compared to $Gaa^{+/+}Cd4^{-/-}$PBS. (D-E) GAA uptake and glycogen clearance in muscles at sacrifice (day 66). (D) Analysis of GAA in muscles at the end of the study. Asterisks (*) indicate significant differences compared to $Gaa^{-/-}Cd4^{-/-}$ PBS, hash symbols (#) indicate significant differences compared to $Gaa^{-/-}Cd4^{-/-}$ AAV-GAA $1x10^{11}$vg/kg (G) Analysis of glycogen content in muscles. Asterisks (*) indicate significant differences compared to $Gaa^{+/+}Cd4^{-/-}$ PBS; hash symbols (#) indicate significant differences compared to $Gaa^{-/-}Cd4^{-/-}$ AAV-GAA $1x10^{11}$vg/kg. All data are shown as mean ± SD. Statistical analysis: One-way ANOVA with Tukey post-hoc test (and # p<0.05, ** and ## p<0.01, ### p<0.001, **** and #### p<0.0001, n = 8 mice per group).*

Next, we measured GAA activity in muscle tissues. GAA activity levels were significantly elevated in heart and skeletal muscles of $Gaa^{-/-}Cd4^{-/-}$ mice receiving the combination AAV-GAA vector and PCs (**Figure 5.6D**). Furthermore, upon a 2-month treatment, while only partial correction of glycogen content was observed in heart and skeletal muscles of $Gaa^{-/-}Cd4^{-/-}$ mice treated by unique injection of AAV-GAA, a complete normalization was observed in heart, diaphragm and quadriceps of mice treated by combination therapy of AAV-GAA and PCs (**Figure 5.6E**).

At the vector dose used for this study ($1x10^{11}$ vg/kg) and upon a 2-month treatment, GT was not effective enough to deliver sufficient GAA to correct refractory tissues like spinal cord and brain. Indeed, despite a slight increase of GAA uptake in spinal cord observed in GSD

II mice treated with PCs, this was not accompanied with a reduction of glycogen accumulation (**Figure 5.7A-B**).

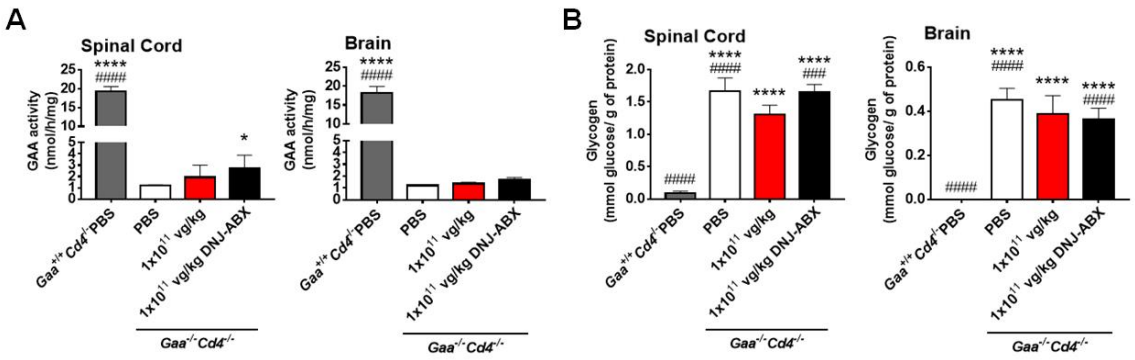


Figure 5.7 GAA uptake and glycogen content in central nervous system.
(A-B) GSD II mice were treated as described in Figure 1.4 (n=8/group). (A) Analysis of GAA uptake in CNS at sacrifice (day 66). Asterisks (*) indicate significant differences compared to Gaa$^{-/-}$Cd4$^{-/-}$ PBS; hash symbols (#) indicate significant differences compared to Gaa$^{-/-}$Cd4$^{-/-}$AAV-GAA 1x10^{11} vg/kg (B) Analysis of glycogen content in CNS. Asterisks (*) indicate significant differences compared to Gaa$^{+/+}$Cd4$^{-/-}$ PBS; hash symbols (#) indicate significant differences compared to Gaa$^{-/-}$Cd4$^{-/-}$ AAV-GAA 1x10^{11} vg/kg.

Taken together, the results obtained show that oral administration of PCs (DNJ-ABX) enhanced GT efficacy and provided a superior correction of the skeletal muscle disease in GSD II mice over a 2-months follow-up.

4. Discussion - conclusion

Since 2006, the only treatment available for PD is ERT, with rhGAA, approved both in the US and Europe.[14] With a nearly five-fold reduction in mortality rate compared with untreated patients, this drug has profoundly transformed the lives of infantile onset PD (IOPD) patients.[15] On the other hand, skeletal muscle response is much less impressive: modest or no improvement were observed in some patients, while others continue to decline despite the therapy.[16-22] Despite ERT, PD remains lethal for many severely affected subjects.[15, 23, 24] Even when ERT is effective, patients remain weak and continue to carry a heavy burden. Furthermore, ERT does not allow to clear neuronal glycogen, and consequently does not prevent the pathology progression and functional decline, in particular in IOPD patients.[25-28] Additionally, antibody responses to rhGAA mitigate the efficacy of ERT.[29] For all these reasons, despite being currently crucial for the management of PD, ERT with rhGAA is not a cure.

Several appraoches are explored to overcome ERT limitations. New-generation ERTs are currently being tested in clinic such as the Neo-GAA[30] (Genzyme-Sanofi, NCT02032524, NCT03019406, NCT02782741), the ATB200[31] (Amicus Therapeutics, NCT03729362), and the VAL-1221 (Valerion, NCT02898753)[32], trying to improve the delivery, effectiveness and tolerability of ERT. Interesting adjunctive treatments to ERT such as salbutamol (NCT01885936)[33-35] or clambuterol (NCT01942590),[36] are also currently being tested in clinic. First results seem to indicate biochemical improvements with increased GAA activity and decreased glycogen in muscle biopsy, and clinical improvements like improved motor and pulmonary functions in patients under salbutamol/clenbuterol compared to those who received the placebo.

Among the strategies employed to facilitate the GAA delivery, uptake and trafficking, combination of ERT and pharmacological chaperone therapy (PCT) is an attractive approach. After promising results obtained in patients fibroblasts and in Pompe mice,[37-40] Amicus Therapeutics is currently developing this technology in a Phase 1/2 clinical trial (NCT02675465), combining the novel rhGAA ATB200 with a chaperone molecule called AT2221 (NB-DNJ, miglustat) and has recently announced positive results.[41-43]

Chaperone therapy relies on small molecules (often enzyme inhibitors) that, when administered at suboptimal concentrations, bind to the dysfunctional GAA enzyme, helping it to fold correctly, increasing its stability and transit through the Golgi and the lysosomes, thus ensuring that normal enzyme activity is restored (more details in **chapter 1**). While effective, PCT alone is limited because not all variants of the GAA gene mutation described in Pompe patients may respond to this mechanism of action.[9, 44-47] However, used in combination, PCT is able to enhance ERT effectiveness. In the last decade, iminosugar derivatives of DNJ, that are alpha-glucosidase inhibitors, were extremly investigated and efforts have paid off: these molecules were described to be able to promote GAA transport, stability and activity and improve muscle function in animal models.[6-9, 37-40, 44-48] Positive effects of molecules such as N-acetylcysteine (NAC),[49] ABX,[10] but also acarbose,[50] miglitol,[51] voglibose,[4, 52] and new class of α-glucosidase inhibitors names α-cyclosulfamidates,[53] were reported as a consequence of the stabilization of rhGAA, its lysosomal localization increase and enhanced enzyme activity.

Interestingly, although PCs might increase the effectiveness of ERT, to our knowledge none of them, was evaluated in combination with GT. We have shown that hepatic AAV8 GT with engineered secretable GAA significantly reduces glycogen accumulation in muscle and CNS and rescues the disease phenotype of adult symptomatic *Gaa*$^{-/-}$ mice upon a single vector administration at vector doses from $5x10^{11}$ to $2x10^{12}$vg/kg.[1-3, 54] Then, we have also shown hepatic secretion of engineered GAA into the circulation and uptake in key tissues affected by PD in NHP,[1] a fundamental step for the clinical development of hepatic AAV-GAA GT. As compared to ERT, this GT approach has the inherent advantage to possibly be a once-in-a-life treatment, as observed in clinical trials of hepatic AAV gene transfer for Hemophilia B, which is caused by the deficiency of coagulation Factor IX.[55-57] Furthermore, as recently reported in a PD mouse model, this strategy seems to show a superior therapeutic efficacy than ERT.[3]

In the present work, we were particularly interested to test PCT in combination with GT and investigate if and how PCs could enhance activity and tissue uptake of AAV-mediated hGAA. Among the molecules that we tested, we could not investigate the AT2221, which is currently clinically tested and which seems to be promising. Further experiments in order to evaluate the combination AT2221 and GT will give valuable indications on the potential of the PCT. Here, we used the compound DNJ hydrochloride (AT2220) and tried to associate it with ambroxol hydrochloride (ABX) in order to potentiate its effects, as previously demonstrated by others *in vitro*.[10] Since the last years, ABX has been extensively studied in the context of GBA dysfunctions in Gaucher disease[58, 59] or Parkinson disease[60-66] but also for other neurodegenerative diseases such as familial amyotrophic lateral sclerosis[67, 68] and it is defined as a safe molecule able to cross the blood–brain barrier (BBB) and to improve lysosomal biochemistry. ABX is described as a chaperone able to modulate the glucocerebrosidase activity,[69] stimulate axonal plasticity and the formation of neuromuscular junctions and also interact with neuroprotective factors.[67, 68] This potential neuroprotective effect in motor neuron diseases or lysosomal storage disorders deserves to be studied in detail. To make full use of these specific properties, we decided to test it as a

chaperone for GAA, with the goal to improve the therapeutic efficacy of AAV-GAA GT in nervous tissues.

In this study, we first demonstrated positive effect of one month of PCT on levels and activity of circulating hGAA in AAV-GAA-treated wild-type mice. We observed in those mice up to a ~1.5 fold increase of hGAA activity and a ~3 fold increase of hGAA levels, compared to mice treated with GT alone. These results were consistent with the improvement of ERT efficacy described in the literature with those molecules.[6, 8] We also confirmed here the works of Lukas et al.,[10] and demonstrated, in a therapeutically relevant animal model, the capability of the combination DNJ-ABX to improve rhGAA bioavailability, as shown by an ~1.5 fold increase of rhGAA activity and a ~2 fold increase of rhGAA levels in the blood of ERT-treated mice.

One month PCT enhanced hGAA uptake in muscle, especially in triceps and in diaphragm of AAV-treated GSD II mice. A clear advantage of the combination of DNJ and ABX used as PCT was observed in the spinal cord. Indeed, we measured significantly higher levels of hGAA in this nervous tissue in mice treated with these PCs than in all other cohorts. We have associated these results with the intrinsic property of ABX to reach this tissue. In PD, accumulation of glycogen in the CNS was shown to contribute to severe muscle weakness, and both skeletal muscle- and CNS-targeting therapies are needed to fully correct the phenotype.[70] Notably, diaphragm stimulation defects were reported in PD subjects,[71] suggesting that the correction of the pathological consequences of glycogen accumulation in this muscle would be not sufficient to improve the respiratory function in the absence of concomitant correction of afferent phrenic motor neurons in the spinal cord. The data of the present work suggest that PCs, especially the combination DNJ-ABX, may offer an advantage for treating the CNS manifestations of PD. On the other hand, we did not observe improvement of hGAA uptake in brain, despite reported capacity of DNJ and ABX to cross the BBB in mice.[7, 64] It must be noted, however, that results pertaining to BBB penetration efficiency are often difficult to interpret, notably due to strain- and species-specific differences, already reported for example in AAV vector delivery.[72, 73] In addtion, further investigations with posology adjustments of PCs (doses, frequency, gavage vs. dilution in drinking water) could help to ameliorate these results. For example, Khanna et al. suggested that greater PCT efficacy could be achieved using less-frequent AT2220 administration regimens and lower doses (30mg/kg) to avoid any GAA inhibition.[7, 74] As reported by D'Alonzo and colleagues, other ameliorations could also be brought by the use of slightly modified PCs, capable of increasing GAA levels in the lysosomes without inhibiting enzyme function when co-administered with rGAA.[48]

Next, we confirmed in GAA deficient mice the encouraging data obtained in wild-type mice. In this work, we deliberately chose to treat the mice with a suboptimal AAV vector dose ($1x10^{11}$vg/kg) to better see if PCs could help to ameliorate the whole-body rescue of PD phenotype. Indeed, the risk of using higher vector doses was to correct the glycogen accumulation (as we demonstrated in Pompe mice at vector doses starting from $5x10^{11}$vg/kg)[1-3, 54] Even at the very low vector dose tested here, significant glycogen reduction in muscle (heart, diaphragm, quadriceps, and triceps) and improvement of muscle strength were shown. A better improvement of the disease phenotype was reported in GSD II mice treated by the combined therapy of DNJ-ABX and AAV. Notably, we observed higher activity and higher levels of circulating hGAA, associated with higher expression in the liver. While we showed later that PCs administration do not seem to impact liver transduction efficacy, the liver VGCN in this experiment was superior in mice treated with PCT. We do not have a clear explanation to this increase and this is an important question which should be explored further. Still, these molecules clearly increased the GT efficacy.

An overall better clearance of glycogen was observed after PCT as compared to GT alone. After two months of combined treatment, heart glycogen accumulation and cardiac hypertrophy were normalized and glycogen accumulation in muscle was corrected, similarly to what observed after administration of higher AAV vector doses.[1-3, 54]

Unfortunately and in consistence with our previous works,[1-3, 54] while we were able to detect small levels of lysosomal GAA in the spinal cord and the brain of GSD II mice administered with AAV-GAA ($1x10^{11}$vg/kg), they were not sufficient to reduce glycogen storage in these tissues as compared to control affected mice. Short PCT had no effect on these nervous tissues surely because of the small amount of hGAA taken up by these tissues at the very low dose used. More investigations and optimizations of the combination therapy that we proposed here are still needed to improve the correction of the neuropathological component of PD.

To our knowledge, the present work is the first to study the association of the PCT and GT in the context of PD. We showed a superior therapeutic efficacy of this combined therapy in a PD mouse model as compared to GT alone. This was due to an improved circulating enzyme availability and uptake in tissues, especially in skeletal muscles. As reported by many studies, even minimal increases in activity may be sufficient to positively impact the phenotype. In particular, it has been assumed that a threshold activity of ~10% is sufficient to prevent storage in several LSDs, with restoration of 3–5% activity often cited as sufficient to slow down the disease progression.[44, 75-77] This suggests that the enhancement of GAA activity observed in our study with PCT could be very beneficial for the patients.

Altogether, our findings represent a very promising approach for the development of a more effective hepatic AAV GT with secretable GAA for PD and motivate for further explorations. PCT might increase the safety and tolerability of AAV vectors and ameliorate the biodistribution of the GAA, including in the CNS. In particular, this strategy combined with high vector optimization may help to reduce the vector dose to administer to patients, reducing as well the potential related immuno-toxicity.[78] In conclusion our study provides encouraging preliminary results of a novel therapeutic modality for PD that still needs improvement to efficiently clear glycogen in multiple tissues including refractory muscle and nervous tissues.

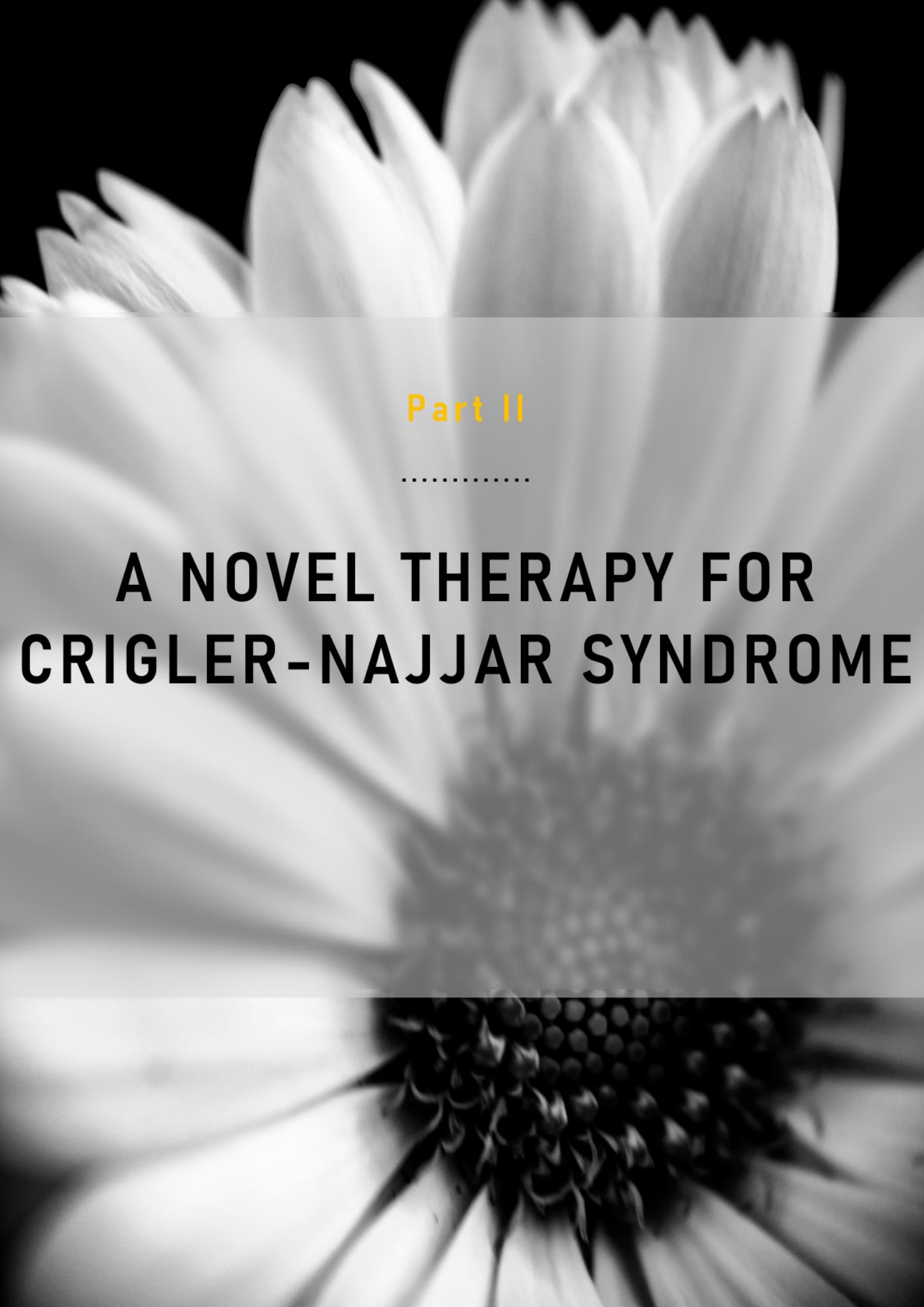
5. References

1. Puzzo, F. et al. Rescue of Pompe disease in mice by AAV-mediated liver delivery of secretable acid alpha-glucosidase. *Sci Transl Med* **9** (2017).
2. Cagin, U. et al. Rescue of advanced Pompe disease in mice with hepatic expression of secretable acid α-glucosidase. *Molecular Therapy* (2020).
3. Armour, S. et al. O. 6Pre-clinical development of SPK-3006, an investigational liver-directed AAV gene therapy for the treatment of Pompe disease. *Neuromuscular Disorders* **29**, S39 (2019).
4. Bruckmann, C., Repo, H., Kuokkanen, E., Xhaard, H. & Heikinheimo, P. Systematic Structure–Activity Study on Potential Chaperone Lead Compounds for Acid α-Glucosidase. *ChemMedChem* **7**, 1943-1953 (2012).
5. Bellotti, A.S. et al. Molecular Approaches for the Treatment of Pompe Disease. *Molecular neurobiology* **57**, 1259-1280 (2020).
6. Khanna, R. et al. The pharmacological chaperone AT2220 increases recombinant human acid α-glucosidase uptake and glycogen reduction in a mouse model of Pompe disease. *PloS one* **7**, e40776 (2012).
7. Khanna, R. et al. The pharmacological chaperone AT2220 increases the specific activity and lysosomal delivery of mutant acid alpha-glucosidase, and promotes glycogen reduction in a transgenic mouse model of Pompe disease. *PLoS One* **9**, e102092 (2014).

8. Kishnani, P. et al. Duvoglustat HCl increases systemic and tissue exposure of active acid α-glucosidase in Pompe patients co-administered with alglucosidase α. *Molecular Therapy* **25**, 1199-1208 (2017).
9. Flanagan, J.J. et al. The pharmacological chaperone 1-deoxynojirimycin increases the activity and lysosomal trafficking of multiple mutant forms of acid alpha-glucosidase. *Human mutation* **30**, 1683-1692 (2009).
10. Lukas, J. et al. Enzyme enhancers for the treatment of Fabry and Pompe disease. *Molecular Therapy* **23**, 456-464 (2015).
11. Ayuso, E., Mingozzi, F. & Bosch, F. Production, purification and characterization of adeno-associated vectors. *Curr Gene Ther* **10**, 423-36 (2010).
12. Raben, N. et al. Targeted disruption of the acid alpha-glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen storage disease type II. *J Biol Chem* **273**, 19086-92 (1998).
13. Zhang, P. et al. Immunodominant liver-specific expression suppresses transgene-directed immune responses in murine pompe disease. *Hum Gene Ther* **23**, 460-72 (2012).
14. Cupler, E.J. et al. Consensus treatment recommendations for late-onset Pompe disease. *Muscle & nerve* **45**, 319-333 (2012).
15. Schoser, B. et al. Survival and long-term outcomes in late-onset Pompe disease following alglucosidase alfa treatment: a systematic review and meta-analysis. *Journal of neurology* **264**, 621-630 (2017).
16. Harlaar, L. et al. Large variation in effects during 10 years of enzyme therapy in adults with Pompe disease. *Neurology* **93**, e1756-e1767 (2019).
17. Stepien, K.M., Hendriksz, C.J., Roberts, M. & Sharma, R. Observational clinical study of 22 adult-onset Pompe disease patients undergoing enzyme replacement therapy over 5 years. *Molecular Genetics and Metabolism* **117**, 413-418 (2016).
18. Van der Ploeg, A.T. et al. Open-label extension study following the Late-Onset Treatment Study (LOTS) of alglucosidase alfa. *Molecular genetics and metabolism* **107**, 456-461 (2012).
19. van der Ploeg, A.T. et al. A randomized study of alglucosidase alfa in late-onset Pompe's disease. *N Engl J Med* **362**, 1396-406 (2010).
20. Ebbink, B.J. et al. Cognitive decline in classic infantile Pompe disease: An underacknowledged challenge. *Neurology* **86**, 1260-1261 (2016).
21. Prater, S.N. et al. The emerging phenotype of long-term survivors with infantile Pompe disease. *Genetics in medicine* **14**, 800-810 (2012).
22. van den Dorpel, J. et al. Distal muscle weakness is a common and early feature in long-term enzyme-treated classic infantile Pompe patients. *Orphanet Journal of Rare Diseases* **15**, 1-9 (2020).
23. Kazi, Z.B. et al. Sustained immune tolerance induction in enzyme replacement therapy-treated CRIM-negative patients with infantile Pompe disease. *JCI Insight* **2** (2017).
24. Kishnani, P.S. et al. Recombinant human acid [alpha]-glucosidase: major clinical benefits in infantile-onset Pompe disease. *Neurology* **68**, 99-109 (2007).
25. Elmallah, M.K. et al. Sustained correction of motoneuron histopathology following intramuscular delivery of AAV in pompe mice. *Mol Ther* **22**, 702-12 (2014).
26. Falk, D.J. et al. Comparative impact of AAV and enzyme replacement therapy on respiratory and cardiac function in adult Pompe mice. *Mol Ther Methods Clin Dev* **2**, 15007 (2015).
27. DeRuisseau, L.R. et al. Neural deficits contribute to respiratory insufficiency in Pompe disease. *Proc Natl Acad Sci U S A* **106**, 9419-24 (2009).
28. Korlimarla, A., Lim, J.-A., Kishnani, P.S. & Sun, B. An emerging phenotype of central nervous system involvement in Pompe disease: from bench to bedside and beyond. *Annals of Translational Medicine* **7** (2019).
29. Patel, T.T. et al. The impact of antibodies in late-onset Pompe disease: a case series and literature review. *Molecular genetics and metabolism* **106**, 301-309 (2012).
30. Pena, L.D. et al. Safety, tolerability, pharmacokinetics, pharmacodynamics, and exploratory efficacy of the novel enzyme replacement therapy avalglucosidase alfa (neoGAA) in treatment-naïve and alglucosidase alfa-treated patients with late-onset Pompe disease: A phase 1, open-label, multicenter, multinational, ascending dose study. *Neuromuscular Disorders* **29**, 167-186 (2019).
31. Gotschall, R. et al. Novel recombinant human acid α-glucosidase with optimal glycosylation is significantly better than standard of care enzyme replacement for glycogen clearance in skeletal muscles of GAA knock-out mice. *Molecular Genetics and Metabolism* **2**, S49 (2015).
32. Kishnani, P. et al. Safety and efficacy of VAL-1221, a novel fusion protein targeting cytoplasmic glycogen, in patients with late-onset Pompe disease. *Molecular Genetics and Metabolism* **126**, S85-S86 (2019).
33. Koeberl, D.D. et al. Improved muscle function in a phase I/II clinical trial of albuterol in Pompe disease. *Molecular Genetics and Metabolism* **129**, 67-72 (2020).

34. Koeberl, D.D. et al. Adjunctive albuterol enhances the response to enzyme replacement therapy in late-onset Pompe disease. *The FASEB Journal* **28**, 2171-2176 (2014).
35. Chien, Y.-H. et al. Albuterol as an adjunctive treatment to enzyme replacement therapy in infantile-onset Pompe disease. *Molecular genetics and metabolism reports* **11**, 31-35 (2017).
36. Koeberl, D.D. et al. Correction of biochemical abnormalities and improved muscle function in a phase I/II clinical trial of clenbuterol in Pompe disease. *Molecular Therapy* **26**, 2304-2314 (2018).
37. Porto, C. et al. The pharmacological chaperone N-butyldeoxynojirimycin enhances enzyme replacement therapy in Pompe disease fibroblasts. *Mol Ther* **17**, 964-71 (2009).
38. Parenti, G. et al. A chaperone enhances blood alpha-glucosidase activity in Pompe disease patients treated with enzyme replacement therapy. *Mol Ther* **22**, 2004-12 (2014).
39. Xu, S. et al. Improved efficacy of a next-generation ERT in murine Pompe disease. *JCI Insight* **4** (2019).
40. Meena, N.K., Ralston, E., Raben, N. & Puertollano, R. Enzyme replacement therapy can reverse pathogenic cascade in Pompe disease. *Molecular Therapy-Methods & Clinical Development* **18**, 199-214 (2020).
41. Clemens, P.R. et al. Safety and efficacy of advanced and targeted acid α-glucosidase (AT-GAA)(ATB200/AT2221) in ERT-switch nonambulatory patients with Pompe disease: preliminary results from the ATB200-02 trial. *Molecular Genetics and Metabolism* **126**, S40-S41 (2019).
42. Kishnani, P. et al. First-in-human study of advanced and targeted acid α-glucosidase (AT-GAA)(ATB200/AT2221) in patients with Pompe disease: preliminary functional assessment results from the ATB200-02 trial. *Molecular Genetics and Metabolism* **126**, S86 (2019).
43. Schoser, B. et al. Preliminary patient-reported outcomes and safety of advanced and targeted acid α-glucosidase (AT-GAA (ATB200/AT2221) in patients with Pompe disease from the ATB200-02 trial. *Molecular Genetics and Metabolism* **126**, S132-S133 (2019).
44. Parenti, G., Andria, G. & Valenzano, K.J. Pharmacological chaperone therapy: preclinical development, clinical translation, and prospects for the treatment of lysosomal storage disorders. *Molecular Therapy* **23**, 1138-1148 (2015).
45. MUGRAGE, B. (ed. AMICUS THERAPEUTICS, I.) (2006).
46. Parenti, G. et al. Pharmacological enhancement of mutated α-glucosidase activity in fibroblasts from patients with Pompe disease. *Molecular Therapy* **15**, 508-514 (2007).
47. Okumiya, T. et al. Chemical chaperones improve transport and enhance stability of mutant alpha-glucosidases in glycogen storage disease type II. *Mol Genet Metab* **90**, 49-57 (2007).
48. D'Alonzo, D. et al. N-Butyl-l-deoxynojirimycin (l-NBDNJ): Synthesis of an Allosteric Enhancer of α-Glucosidase Activity for the Treatment of Pompe Disease. *Journal of medicinal chemistry* **60**, 9462-9469 (2017).
49. Porto, C. et al. Pharmacological enhancement of α-glucosidase by the allosteric chaperone N-acetylcysteine. *Molecular Therapy* **20**, 2201-2211 (2012).
50. Jack, R.M., Gordon, C., Scott, C., Kishnani, P.S. & Bali, D. The use of acarbose inhibition in the measurement of acid alpha-glucosidase activity in blood lymphocytes for the diagnosis of Pompe disease. *Genetics in Medicine* **8**, 307-312 (2006).
51. Campbell, L.K., Baker, D.E. & Campbell, R.K. Miglitol: assessment of its role in the treatment of patients with diabetes mellitus. *Annals of Pharmacotherapy* **34**, 1291-1301 (2000).
52. Chen, X., Zheng, Y. & Shen, Y.V. one of the most important α-glucosidase inhibitors. *Curr. Med. Chem* **13**, 109-116 (2006).
53. Herman S. OVERKLEEFT, G.J.D., Johannes M.F.G. Aerts, Marta Elena ARTOLA PÉREZ DE AZANZA, Rhianna J. ROWLAND, Liang Wu. (ed. Leiden University, U.O.Y.) (2020).
54. Colella, P. et al. AAV Gene Transfer with Tandem Promoter Design Prevents Anti-transgene Immunity and Provides Persistent Efficacy in Neonate Pompe Mice. *Mol Ther Methods Clin Dev* **12**, 85-101 (2019).
55. George, L.A. et al. Hemophilia B gene therapy with a high-specific-activity factor IX variant. *New England Journal of Medicine* **377**, 2215-2227 (2017).
56. Nathwani, A.C. et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* **371**, 1994-2004 (2014).
57. Nathwani, A.C. et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med* **365**, 2357-65 (2011).
58. Maegawa, G.H. et al. Identification and characterization of ambroxol as an enzyme enhancement agent for Gaucher disease. *Journal of Biological Chemistry* **284**, 23502-23516 (2009).
59. Narita, A. et al. Ambroxol chaperone therapy for neuronopathic Gaucher disease: a pilot study. *Annals of clinical and translational neurology* **3**, 200-215 (2016).
60. Albin, R.L. & Dauer, W.T. Magic shotgun for Parkinson's disease? *Brain* **137**, 1274-1275 (2014).
61. McNeill, A. et al. Ambroxol improves lysosomal biochemistry in glucocerebrosidase mutation-linked Parkinson disease cells. *Brain* **137**, 1481-1495 (2014).

62. Ambrosi, G. et al. Ambroxol-induced rescue of defective glucocerebrosidase is associated with increased LIMP-2 and saposin C levels in GBA1 mutant Parkinson's disease cells. *Neurobiology of disease* **82**, 235-242 (2015).
63. Migdalska-Richards, A., Daly, L., Bezard, E. & Schapira, A.H. Ambroxol effects in glucocerebrosidase and α-synuclein transgenic mice. *Annals of neurology* **80**, 766-775 (2016).
64. Migdalska-Richards, A., Ko, W.K.D., Li, Q., Bezard, E. & Schapira, A.H. Oral ambroxol increases brain glucocerebrosidase activity in a nonhuman primate. *Synapse* **71**, e21967 (2017).
65. O'Regan, G., deSouza, R.-M., Balestrino, R. & Schapira, A.H. Glucocerebrosidase mutations in Parkinson disease. *Journal of Parkinson's disease* **7**, 411-422 (2017).
66. Mullin, S. et al. Ambroxol for the treatment of patients with Parkinson disease with and without glucocerebrosidase gene mutations: a nonrandomized, noncontrolled trial. *JAMA neurology* **77**, 427-434 (2020).
67. Bouscary, A. et al. Ambroxol Hydrochloride Improves Motor Functions and Extends Survival in a Mouse Model of Familial Amyotrophic Lateral Sclerosis. *Frontiers in pharmacology* **10**, 883-883 (2019).
68. Bouscary, A. & Quessada, C. Potentiel thérapeutique de l'ambroxol contre les maladies du motoneurone. *médecine/sciences* **36**, 447-448 (2020).
69. Shanmuganathan, M. & Britz-McKibbin, P. Inhibitor screening of pharmacological chaperones for lysosomal β-glucocerebrosidase by capillary electrophoresis. *Analytical and bioanalytical chemistry* **399**, 2843-2853 (2011).
70. Byrne, B.J. et al. Pompe disease gene therapy. *Human molecular genetics* **20**, R61-R68 (2011).
71. Smith, B.K., Corti, M., Martin, A.D., Fuller, D.D. & Byrne, B.J. Altered activation of the diaphragm in late-onset Pompe disease. *Respiratory physiology & neurobiology* **222**, 11-15 (2016).
72. Matsuzaki, Y. et al. Intravenous administration of the adeno-associated virus-PHP. B capsid fails to upregulate transduction efficiency in the marmoset brain. *Neuroscience letters* **665**, 182-188 (2018).
73. Hordeaux, J. et al. The Neurotropic Properties of AAV-PHP.B Are Limited to C57BL/6J Mice. *Molecular Therapy* **26**, 664-668 (2018).
74. Adera, M. et al. Pharmacokinetics and muscle distribution of at2220, a pharmacological chaperone of acid-glucosidase, in healthy volunteers. *Molecular Genetics and Metabolism* **102**, S4 (2011).
75. Hopwood, J. & Muller, V. Biochemical discrimination of Hurler and Scheie syndromes. *Clinical Science* **57**, 265-272 (1979).
76. Leinekugel, P., Michel, S., Conzelmann, E. & Sandhoff, K. Quantitative correlation between the residual activity of β-hexosaminidase A and arylsulfatase A and the severity of the resulting lysosomal storage disease. *Human genetics* **88**, 513-523 (1992).
77. Desnick, R.J. & Fan, J.-Q. Pharmacologic chaperone therapy for lysosomal diseases. *GAUCHER* (2006).
78. Mingozzi, F. et al. CD8+ T-cell responses to adeno-associated virus capsid in humans. *Nature medicine* **13**, 419-422 (2007).



Part II

A NOVEL THERAPY FOR CRIGLER-NAJJAR SYNDROME

Chapter 6

Crigler-Najjar syndrome, a model for liver gene therapy

1. Bilirubin Metabolism

Since Hyppocrates and the ancient times of medicine, both liver and bile have always played a central role in the understanding of physiology and anatomy of the human body. However, it took until the 19th century to discover bilirubin, the chemical compound was responsible for the jaundice, this yellow decolourisation of the skin and sclerae, renowned for many centuries as a symptom of illness[1]. Since 2000s, the all pathway of bilirubin metabolism and secretion is known (**Figure 6.1**).

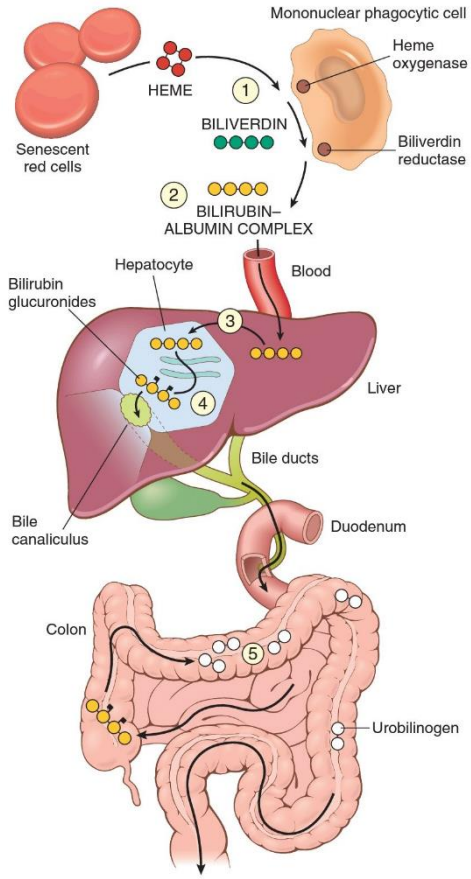


Figure 6.1 Bilirubin metabolism and elimination.
Bilirubin is produced primarily from the breakdown of senescent circulating erythrocyte, and is the end product of heme degradation. 1) Heme is degraded to biliverdin by heme oxygenase in the mononuclear phagocytes and subsequently reduced to bilirubin by biliverdin reductase. 2) Circulating bilirubin (insoluble) is bound to plasma proteins (mainly albumin) and transported to the liver. 3) After dissociation from albumin, bilirubin is delivered inside hepatocytes via a mechanism involving carrier-mediated membrane transport. 4) Inside hepatocytes, bilirubin is bound to cytosolic proteins, such as glutathione-transferases, and is presented for conjugation. Glucuronidation happens there and generates bilirubin monoglucuronides and diglucuronides, which are water soluble and readily excreted into bile. 5) After secretion of conjugated bilirubin in the bile, gut bacteria deconjugate the bilirubin and degrade it to colorless urobilinogen. This urobilinogen and the residues of intact pigments are excreted in the feces, with some reabsorption and re-excretion into bile. Approximately 20% of the urobilinogen are reabsorbed in the ileum and colon, returned to the liver, and re-excreted into bile. Conjugated and unconjugated bile acids are also reabsorbed in the ileum and returned to the liver by enterohepatic circulation.

Bilirubin is a yellow-orange colored bile pigment. The term *bilirubin* is derived from the Latin words *bilis* (bile) and *ruber* (red). It is the end product of degradation of the heme moiety of hemoproteins. In humans, the heme catabolism allows the synthesis of 250 to 400mg of bilirubin per day. The major source, 80%, comes from the degradation of senescent erythrocytes, and the remaining 20% is derived from other hemoproteins of the liver and other organs, such as myoglobin, peroxidase, cytochromes and catalase.[2]

Heme is first degraded by the microsomal heme oxygenase into molecules of ferrous iron, carbon monoxide and biliverdin, by a reaction requiring oxygen and a reducing agent, such as nicotinamide adenine dinucleotide phosphate (NADPH).[3] Then, biliverdin is reduced to unconjugated bilirubin (UCB) by the action of two biliverdin reductases (**Figure 6.2**). As an hydrophobic molecule, UCB has affinity for fat and can diffuse across the BBB into the CNS where it is deleterious. Albumin binding keeps UCB in solution and prevents its diffusion into tissues and all its toxic effects. Why the nontoxic, water-soluble biliverdin is converted to the potentially toxic and hydrophobic

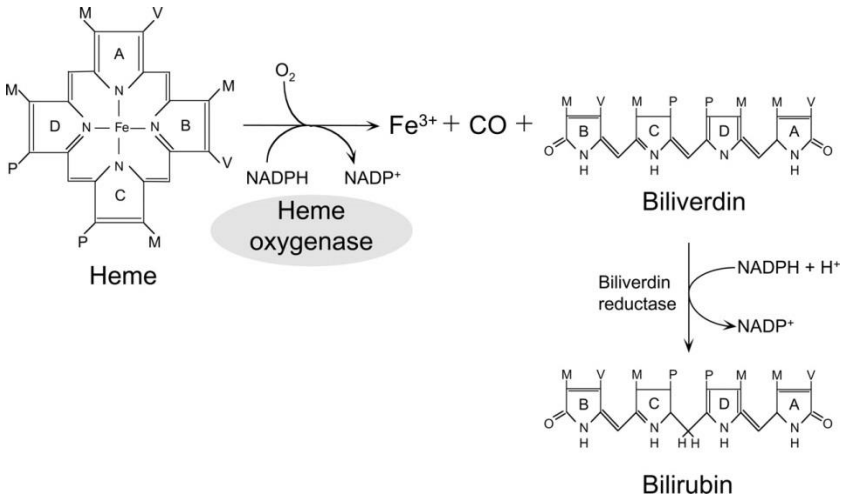


Figure 6.2 Heme oxygenase (HO)-catalyzed reaction.
Heme (iron protoporphyrin IX) is cleaved between rings A and B by heme oxygenase to yield equimolar quantities of iron (Fe3+), carbon monoxide (CO), and biliverdin. O2 and NADPH are required for this reaction. Biliverdin is then converted to bilirubin by biliverdin reductase. M, V, and P: methyl, vinyl, and propionyl groups, respectively. Reproduced from Agarwal et al., 2000 [4]

UCB is still today unclear. One possible explanation could be the facilitated excretion of the UCB in the fetus via the lipophilic placenta.[5] Also, UCB is a potent antioxidant, which may have beneficial effects in neonates.[6]

To allow its excretion, bilirubin has no other choice than to become water-soluble. This is accomplished by glucuronidation with the help of the microsomal UDP glucuronosyltransferase (UGT) family 1 member A1 (UGT1A1). Basically, UCB circulates in the blood bound to albumin. Albumin acts as a buffer for fluctuations of serum bilirubin levels. Important to note, a number of metabolites and drugs can affect this binding capacity and thereby increase the risk of UCB neurotoxicity. At the sinusoidal surface of the liver, UCB is released from albumin and is taken in charge by the hepatocyte, probably via the OATP2 transporter (also known under the name SLC21A6), but this transport of UCB is still not yet clearly identified (**Figure 6.3**).[7, 8]

Once in the hepatocyte, bilirubin is linked to cytosolic proteins, mainly to ligandin, a member of the family of glutathione-transferases (GSTs), which decreases the intracellular bilirubin concentration and prevents UCB from re-entering the circulation. The next step involves conjugation of UCB. Indeed, this chemical transformation is required since only water-soluble bilirubin can be efficiently excreted into the bile. Therefore, UGT1A1 catalyzes the transfer of glucuronic acid from UDP-glucuronate to UCB, generating conjugated bilirubin (CB) under the form of bilirubin mono- and diglucuronides.[9] This glucuronidation makes bilirubin water soluble, so reduces its toxicity, and promotes its secretion into the bile. This step is very crucial. Any reduction of hepatic bilirubin glucuronidating activity results in pathologic build-up of UCB, that is extremly dangerous (see later).

Then, the bilirubin glucuronides produced in periportal hepatocytes (hepatocytes that are initially exposed to bilirubin in portal blood) are mainly delivered into the bile canaliculi via an active transport mediated by the pump ABCC2 (also named MRP2), but also substantial part is secreted into the sinusoidal circulation by the hepatic efflux pump ABCC3 (MRP3) Figure **6.3**).[10-13] Flowing toward the central vein, they undergo reuptake via the organic

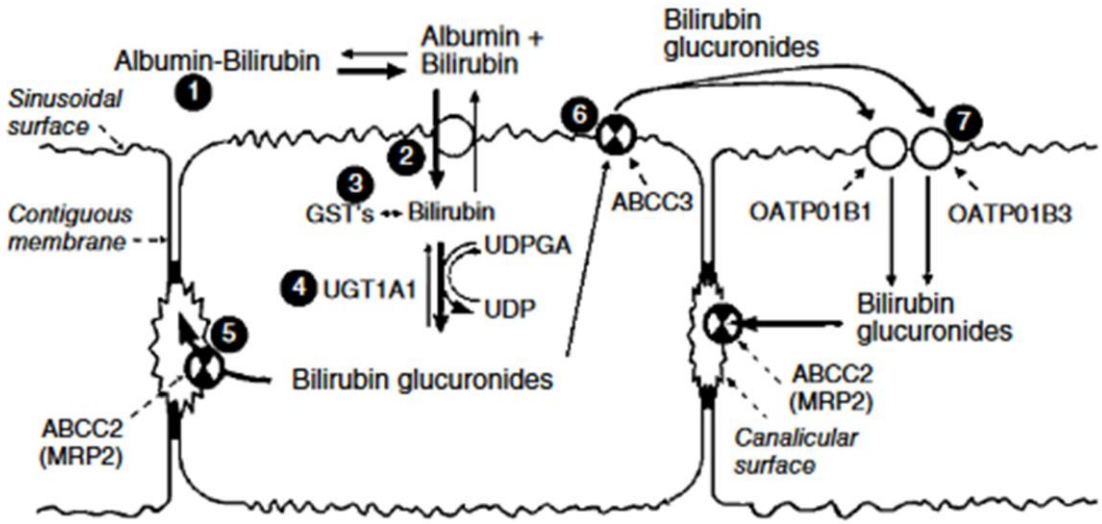


Figure 6.3 Summary of hepatocellular transport and metabolism of bilirubin.
1) Bilirubin is strongly bound to albumin in the circulation. 2) At the sinusoidal surface of the hepatocyte, this complex dissociates, and bilirubin enters hepatocytes by facilitated diffusion. 3) This process is non-adenosine triphosphate (ATP)-dependent and bidirectional. Within the hepatocyte, bilirubin binds to a group of cytosolic proteins, mainly to glutathione-S-transferases (GSTs). GST binding inhibits the efflux of bilirubin from the cell, thereby increasing the net uptake. 4) A specific form of uridine diphosphoglucuronate glucuronosyltransferase (UGT) (termed UGT1A1), located in the endoplasmic reticulum, catalyzes the transfer of the glucuronic acid moiety from UDP-glucuronic acid (UDPGA) to bilirubin, forming bilirubin diglucuronide and monoglucuronide. Glucuronidation is necessary for efficient excretion of bilirubin in bile. 5) Canalicular excretion of bilirubin and other organic anions (except most bile acids) is primarily an energy-dependent process, mediated by the ATP-utilizing transporter multidrug resistance-related protein ABCC2, also known as multidrug-resistance-related proteins (MRP2). 6) Excess bilirubin glucuronides are pumped back into the plasma by ABCC3 located at the sinusoidal membrane and 7) undergo reuptake by hepatocytes located downstream to the portal blood flow via sinusoidal surface organic anion transport proteins, OATP01B1 and OATP01B3.
Reproduced from Chowdhury et al., 2020[14]

anion-transporting polypeptides OATP1B1 (SLC01B1) and OATP1B3 (SLC01B3) located in hepatocyte sinusoidal membranes.[13] Thanks to this process, additional hepatocytes are recruited, thereby increasing the bilirubin-excreting capacity of the liver.

Whe bilirubin glucuronides are introduced in the intestinal tract, they are metabolized by bacterial enzymes into colourless urobilinogen, and then oxidized into an orange compound called urobilin, which will be finally eliminated via feces and urine. A small resistant part of urobilin will be absorbed by the portal blood stream and will go back to the liver via the binding with albumin, to be metabolized again in order to be secreted into the bile. In this way, bilirubin participates in the enterohepatic cycle.

Mechanisms of bilirubin uptake, conjugation, and excretion are closely balanced, so that the reduction of any of these processes limits the bilirubin handling capacity of the liver. In contrast, for instance in response to increased bilirubin load, all these pathways required to be coordinately upregulated. Several nuclear receptor proteins, such as CAR and PXR, may regulate such coordinated regulation.[15-17]

2. Crigler-Najjar syndrome

Accumulation of bilirubin in the blood is called hyperbilirubinemia. It can also accumulate in other tissues, where it can cause a yellow coloration of the skin or the sclerae, referred to as jaundice or icterus. Most dangerous is the accumulation of bilirubin in the brain, that provokes necrosis and apoptosis, which may lead to permanent neurological damages.[18]

Accumulation of bilirubin has many causes but mainly results from an imbalance between bilirubin production and its metabolism and/or excretion. Hyperbilirubinemia can involve both UCB and CB[19]. Since albumin binding abrogates the toxic effect of bilirubin, the harmful effects occur when bilirubin is present in a molar excess over albumin. Such conditions are usually limited to neonates and to people affected by an inherited disorders of bilirubin conjugation. This chapter will be limited to the subject of our research: the most severe form of unconjugated hyperbilirubinemia, resulting from decreased hepatic clearance of bilirubin, the Crigler-Najjar (CN) syndrome.

In 1952, John Crigler and Victor Najjar described "a mysterious illness that caused jaundice and severe neurological damage"[20]. They reported a severe occurrence of jaundice in 6 young patients from the same family and proved the character inherited of this neonatal jaundice. The measured levels of bilirubin in blood were very high (340µM) and all 6 patients died as a result of neurological complications. Future analyses will reveal the presence of UCB in the brain, causing bilirubin encephalopathy – kernicterus.

Severity of unconjugated hyperbilirubinemia is very variable between patients and Dr. Arias was the first in 1969 to discriminate the disease in two groups[21]: he distinguished between patients for whom treatment with phenobarbital had no effect on the hyperbilirubinemia, and those for whom phenobarbital decreased the UCB accumulation in the blood. Besides, he noted that patients from the second group had lower levels of bilirubin in blood and that none has developed kernicterus. He thus suggested the following classification: disease of type I (CN I) for the "non-responder" to phenobarbital patients and type II (CN II) for the others. Since then, the unconjugated inherited hyperbilirubinemia has been called Crigler-Najjar syndrome, and the CN II is also known as Arias syndrome. For long the cause of CN syndrome has been enigmatic but the discovery of UGT1A1 finally demonstrated that it was due to a lack of activity of this enzyme to inactivating mutations in the UGT1A1 geen leading to reduced or absent enzyme activity.[22, 23]

2.1 Signs and symptoms

CN syndrome is rare autosomal inherited recessive disorder of hepatic bilirubin excretion. It is characterized by the deficiency of bilirubin glucuronidation. CN syndrome affects males and females in equal numbers. The incidence is estimated to be 1 in 1million people in the general population.[24-27] It is believed that there is a hundred known patients in Europe, but many physicians guess that the disease is often undiagnosed, making it difficult to determine its true frequency in the general population.

In CN syndrome, the deficiency of UGT1A1-mediated glucuronidation renders excretion into bile virtually impossible, resulting in the accumulation of the neurotoxic UCB in serum.[9, 28] The severity of the clinical symptoms correlates with the degree of residual function of UGT1A1. The hallmark of CN I patients is a complete absence of this enzyme resulting in a life-long, severe non-hemolytic unconjugated hyperbilirubinemia. Untreated, serum bilirubin concentrations would range between 340-425µM but can reach values as high as 850µM. For a comparison, in normal subjects bilirubin levels in serum are below 17µM. Patients with CN II still have residual UGT1A1 activity, which results in a milder phenotype. In these patients, bilirubin levels in serum range between 50 and 340µM. Importantly, these patients are to some extent still able to conjugate and excrete bilirubin via the bile.[29]

The symptoms of CN I become apparent shortly after birth and persist over time. Affected children develop severe jaundice with evident signs of yellowing of the skin, mucous membranes and sclera. These patients are at risk of developing kernicterus during the first months of life. More precisely, this is a neurological condition caused by damage to the central nervous system, due to the toxic levels of UCB accumulated in the brain. Early signs may include the lack of energy, the absence of some reflexes, muscle spasms, vomiting, fever or unsatisfactory feedings. It can also result in severe complications such as mental retardation, hearing loss, convulsions, and involuntary writhing movements of the body.[30, 31] An episode of kernicterus can ultimately result in fatal brain damage. The risk for kernicterus persists into adult life, but the level of UCB and the conditions at which that risk becomes critical are unknown. Serum bilirubin is unconjugated and tightly bound to albumin, therefore bilirubinuria is absent in CN I subjects. The bile contains only small amounts of UCB and bile canalicular transport is normal. Although fecal urobilinogen excretion is reduced, the stool color remains normal.[20, 32] Excepting plugs in bile canaliculi and bile ducts, historically liver histology has been reported as normal. However, recent analysis of CN I subjects showed liver fibrosis of various degrees.[33, 34] This liver fibrosis was not associated with portal hypertension and there was no significant correlation with gallstones.

As previously mentioned, CN II is a milder disorder than a type I. In most cases, it is compatible with a normal life. Affected children develop jaundice as well, but normally present no risk for kernicterus throughout the whole life.[20] In some cases, jaundice may not be apparent except during punctual episodes, such as period of intercurrent illnesses, prolonged fasting or under general anesthesia.[35] Moreover, some cases of CN II have not been detected until adult age. As in CN I, there is no evidence of hemolysis or other liver dysfunction, but in contrast to CN I, bile contains a significant amount of bilirubin glucuronides : bilirubin monoglucuronide exceeds 30% of total CB (normal, ~10%), reflecting a reduced hepatic UGT1A1 activity. Liver histology is normal, and UGT1A1 activity is usually reduced to 10% normal.[36]

2.2 Genetic causes

The gene responsible for the production of the UGT1A1 enzyme and its exact structure were identified in the beginning of 1990s and located on chromosome 2q37.[37-39] This gene belongs to a complex locus which spreads on more than 500kb, encoding for several UGT responsible of conjugation of different substrates (such as estradiol). An alternative splicing allows the creation of 10 to 14 isoforms. The locus is composed of a set of alternate first exons followed by four common exons (**Figure 6.4**). Each first exon is regulated by its own promoter and encodes for the substrate binding, whereas the 4 common exons probably determine the interaction with UDP-glucuronic acid.[40]

Mutations detected in CN patients and *in vitro* activity assays of different isoforms of UGT1A showed that 1A1 isoform was responsible for the hepatic bilirubin glucuronidation activity.[9] CN type I is caused by mutations, including deletions, insertions, and premature stop codons, within the five exons of the *ugt1a1* gene that lead to an inactive form of the enzyme.[41, 42] In type II, the genetic lesions consist exclusively of point mutations and result in an UGT1A1 activity less than 5% of normal. Since 1992, over 130 different mutations of

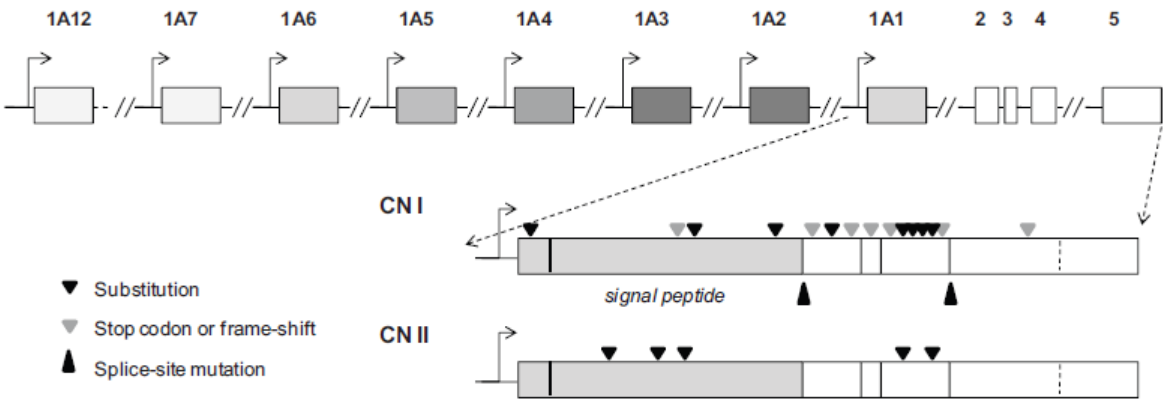


Figure 6.4 Organization of the UGT1A gene
Four common exons (exons 2, 3, 4, and 5) are used in several UGT isoforms expressed from this locus. Upstream from these exons are a series of "unique" exons, only one of which is used in a given isoform. Each unique exon is preceded by a separate promoter (arrows). Splicing of exon 1A1 to the common region exons (2 to 5) generates the mRNA for UGT1A1. Genetic lesions in any of the five exons can abolish (Crigler-Najjar type I) or reduce (Crigler-Najjar type II) the enzyme activity. Adapted from Montenegro Miranda, 2012[43]

ugt1a1 have been identified in CN patients and has been reported.[44, 45] As CN I or CN II can be caused by any of a large number of mutations, no particular one is very common in any ethnic group or community. An exception to this is seen where there is a high level of consanguinity, such as in the Amish–Mennonite community. In this community, the incidence of CN I is very high and all affected people are carrying a specific nonsense mutation in exon 1 of *ugt1a1*.[46]

2.3 Treatment options

To prevent brain toxicity in severe CN patients, the objective is to maintain the serum bilirubin concentration below 350µmol/L. Although several drugs have proved effective to slightly reduce jaundice (e.g. Ursodiol, lipid-rich food, agar, oral calcium carbonate and orlistat reducing bilirubin reuptake in the gut or tin-protoporphyrin and tin-mesoporphyrin inhibiting heme oxygenase activity),[26, 46-49] to date no authorized medicinal product exists for the treatment of CN syndrome. Administration of phenobarbital can enhance the residual UGT1A1 activity in most CN II patients and decrease serum UCB concentrations by approximately 30%, but it is not effective in CN I patients.[21, 29] Importantly, phenobarbital administration provides also a helpful tool to differentiate the two conditions.[50]

In the late 1950s, it was discovered that exposure to sunlight ameliorated hyperbilirubinemia in neonates.[51] The mechanism is imperfectly known but blue light would allow decreasing the bilirubin levels in the blood by converting bilirubin into its photo-isomers, soluble and so secretable into the bile and urine without the need for conjugation.[52-55] This has led to the development of phototherapy (PT). This daily treatment is the mainstay of long-term management for patients with severe CN, but is not required in type II patients, since the serum bilirubin concentration usually remains below 340µM. For an efficient PT preventing encephalopathy (kernicterus) and irreversible neurological sequels, CN I patients need to stay under fluorescent blue light lamps located 20cm from the skin and covering at least 40% of the body area, for 10 to 12 hours per day.[56] These last years, largely due to dedicated families and caregivers, PT has improved a lot. Fluorescent blue lamps are gradually replaced by light-emitting diode (LED) lamps (heating much less) and LED “light blankets” or “light jackets” have been also devised.

Before the introduction of PT, all CN I patients died from kernicterus, but now patients survive with limited or no neurological problems.[24, 57] However, full compliance to this treatment has a great impact on social life, which tends to decrease compliance over time.[58] It also has important shortcomings like a persistent risk of life-threatening spikes of UCB, for instance, due to trauma or sepsis.[30] Furthermore, PT efficiency also decreases with age, due to skin thickening, increased pigmentation, and decreased exposed surface/weight ratio.[46, 59, 60]

During exacerbations of jaundice, due to fasting or concurrent illness for example, several measures in addition to continuous high-intensity PT are taken to manage the disease safely, including albumin infusion (if the bilirubin-albumin molar ratio is above 0.7), exchange transfusion or plasmapheresis.[27, 30, 32] In these situations, medications suceptible to displace bilirubin from albumin are banned and the systematic measurement of unbound plasma bilirubin and the reserve bilirubin binding capacity helps to estimate of the risk of bilirubin-induced neurological damage. This is particularly important in infants, in whom the total plasma bilirubin concentrations used as thresholds for instituting PT and/or exchange transfusion may be misleading.

In most CN I patients, PT is applied only for several years. Actually, despite this intensive treatment, as soon as bilirubin levels reach 400µM, a LTx is preconized to normalize bilirubin levels.[24, 31, 61-63] So far, orthotopic LTx is the only curative treatment for CN I. It allows permanent correction of enzyme deficiency and helps to shelter patients of any neurological complication. Despite the risks in term of morbidity and mortality, it is usually considered before adolescence since some transplant centers advocated that LTx should be performed at a young age to prevent irreversible brain damage.[24, 61, 64] Importantly, patient and family have to take into account the immediate and late complications as well as the long-term complication associated with the graft. Those include surgical problems, transplant rejection, fibrosis of the graft, infections but also, and linked to a long-life immunosuppression treatment, malignancies, renal failure or *de novo* diabetes.[62, 65-67] For instance, up to 20% of transplanted patients develop skin cancer, which is particularly problematic for CN patients who are at higher risk because of their exposure to PT.[67] In fact, there is a delicate balance to find between postpone as much as possible invasive surgery and exposure to immunosuppressive drugs and intervene before a greater risk of kernicterus.[68] In France, patients and physicians are often reluctant to undertake such an irreversible procedure and it is common to postpone transplantation until the therapeutic inadequacy of other treatments is proven.

Although LTx is broadly accepted and mostly successful, several important problems remain such as the limited availability of donor livers. As consequence, irreversible pathology often occurs before LTx is performed. Moreover, this approach still seems disproportionate to correct a single missing enzymatic function in an otherwise normal liver. In addition to that, the estimated 10% mortality risk supports the development of new alternative curative therapies warranted. One experimental option consists in transplanting hepatocytes. After extensive validation in animal model, isolated allogeneic human hepatocytes were transplanted into the liver of CN I patients. [62, 69-71] These studies showed that only 2% of the hepatocyte mass is necessary for a proper conjugation of bilirubin. This corresponds to about 5 to 10% of enzyme activity. Nonetheless, due to immune responses and inefficient grafting of transplanted cells, so far, only a transient partial decrease of bilirubin concentration in blood was reported.[62, 70, 71] Novel strategies are being explored to improve the engraftment and the proliferation of transplanted normal hepatocytes, such as the use of controlled regional irradiation of the liver in combination with a variety of mitotic stimuli, or the use

of HLCs generated by differentiating iPSCs derived by reprogramming somatic cells (e.g. skin fibroblasts, bone marrow cells, peripheral blood mononuclear cells, or epithelial cells shed in the urine).[72-77] Although much less invasive and resulting in a much lower mortality, the need for lifelong immune suppression remains a major drawback for this approach.

Seven decades after its discovery, CN syndrome remains a morbid and potentially fatal disorder and an unmet medical need.[58, 78] In that context, and because it is a monogenetic disorder that does not result in liver damage, liver-directed gene replacement therapy offers the potential of a cure.

2.4 Perspective of gene therapy

CN syndrome seems to be an attractive model disease for the development of a liver gene therapy strategies. One reason is that CN is a well characterized disorder, at molecular and biochemical levels, where 5% of normal activity of the UGT1A1 enzyme is sufficient to reduce the severity of the disease.[79] Next, it is relatively easy to monitor the efficacy of the treatment with standard clinical analyses, e.g. by quantification of bilirubin concentrations in blood or bilirubin glucuronides in bile. CN syndrome does not affect liver histology and does not cause liver damages, legitimating the gene therapy approach. Another reason is also the facilitated access to the relevant preclinical animal models to realize proof-of-concept studies.

In the 1930s (meaning a long time before the first CN patient reported in the literature), the geneticist Charles Kenneth Gunn discovered three young icteric Wistar rats, in a litter of thirteen pups. He noticed that they exhibited a persistent non-hemolytic unconjugated hyperbilirubinemia and that no bilirubin was found in their bile. After multiple cross-breeding experiments, he discovered that this was due to an autosomal recessive mutation.[80, 81] The cause of the jaundice was identified more than 20 years later as a glucuronyl transferase deficiency, avoiding bilirubin conjugation and excretion.[82] These rats are homozygous for a deletion in the common region exon 4, causing a frameshift and a premature stop codon resulting in the expression of a truncated protein. This leads to the loss of activity of all UGT isoforms expressed from the Ugt1 locus. Ever since, the jaundiced “Gunn rat” has played a major role in bilirubin research all over the world, and has contributed to the development of novel cell and gene-based therapies for CN syndrome.[69, 83] This rat model , despite a complete absence of UGT1A1 activity, presents a less severe disease than the human form, comparable to CN type II. More recently, different mouse models containing null mutations and conditional mutations of the Ugt1 gene were generated. [84, 85] These mice display a much severe phenotype than Gunn rats. Indeed, they more closely reproduce the human features of CN I and require PT to survive. These model represents a very useful model to develop and test novel technologies.

Various gene therapy strategies developed during two decades have been applied in animal models of CN, including : i) *ex vivo* gene therapy, based on the transduction of primary hepatocytes using viral vectors, followed by implantation into the liver ; ii) *in vivo* gene replacement using non-viral or viral vectors approaches to directly transduce hepatocytes ; and iii) gene editing for correction of a specific mutation in the UGT1A1 gene, or for insertion of the UGT1A1 gene in a “safe harbor” site of choice, for example to take advantage of a strong endogenous promoter (e.g. albumin). All these different strategies have been largely reviewed in the last years.[43, 86-89]

Among several promising results, AAV-based LGT seems to have most potential in CN syndrome, correcting the phenotype through normalization of bilirubin in rodents. After validation in preclinical experiments, this strategy is currently being tested in a Phase I/II clinical trial (see also Chap. 7).[90, 91]

3. Conclusion

68 years following its discovery, even with existing standards of care, CN syndrome remains a morbid and a life-threatening disease. Despite recent technical advances in CN patients management, brain injury still occurs in 18-25% of subjects in developed countries, and 50-93% in countries where medical resources are scarce. Although LTx represents an effective treatment, it entails well-known surgical, infectious, and malignant risks. Alternative emerging therapies, such as gene therapy, hold promises: they have the potential to reduce the disease burden and improve the lives of CN patients and their families. A true hope for patients, a clinical trial was recently initiated in 4 centers in Europe, led by the CureCN consortium which is composed by researchers, patient's associations, clinicians and biotechnology companies. Its objectives are to assess the safety and tolerance of an AAV-based LGT product, and evaluate the therapeutic efficacy of the candidate drug.

4. References

1. Städeler, G. Ueber die farbstoffe der galle. *Justus Liebigs Ann Chem* **132**, 323-354 (1864).
2. Bissell, D.M. Formation and elimination of bilirubin. *Gastroenterology* **69**, 519-38 (1975).
3. Yoshida, T. & Migita, C.T. Mechanism of heme degradation by heme oxygenase. *Journal of inorganic biochemistry* **82**, 33-41 (2000).
4. Agarwal, A. & Nick, H.S. Renal response to tissue injury: lessons from heme oxygenase-1 GeneAblation and expression. *J Am Soc Nephrol* **11**, 965-73 (2000).
5. Ives, N.K. Rennie and Roberton's Textbook of Neonatology : Neonatal jaundice (1999).
6. Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N. & Ames, B.N. Bilirubin is an antioxidant of possible physiological importance. *Science* **235**, 1043-6 (1987).
7. Cui, Y., Konig, J., Leier, I., Buchholz, U. & Keppler, D. Hepatic uptake of bilirubin and its conjugates by the human organic anion transporter SLC21A6. *J Biol Chem* **276**, 9626-30 (2001).
8. Wang, P., Kim, R.B., Chowdhury, J.R. & Wolkoff, A.W. The human organic anion transport protein SLC21A6 is not sufficient for bilirubin transport. *Journal of Biological Chemistry* **278**, 20695-20699 (2003).
9. Bosma, P.J. et al. Bilirubin UDP-glucuronosyltransferase 1 is the only relevant bilirubin glucuronidating isoform in man. *J Biol Chem* **269**, 17960-4 (1994).
10. Keppler, D., Leier, I. & Jedlitschky, G. Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1 and MRP2. *Biol Chem* **378**, 787-91 (1997).
11. Paulusma, C.C. et al. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* **271**, 1126-8 (1996).
12. JEDLITSCHKY, G. et al. ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. *Biochemical Journal* **327**, 305-310 (1997).
13. Keppler, D. The roles of MRP2, MRP3, OATP1B1, and OATP1B3 in conjugated hyperbilirubinemia. *Drug Metabolism and Disposition* **42**, 561-565 (2014).
14. Chowdhury, N.R., Li, Y. & Chowdhury, J.R. Disorders of bilirubin metabolism. *The liver: biology and pathobiology*, 229-244 (2020).
15. Huang, W. et al. Induction of bilirubin clearance by the constitutive androstane receptor (CAR). *Proceedings of the National Academy of Sciences* **100**, 4156-4161 (2003).
16. Xie, W. et al. Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. *Proceedings of the National Academy of Sciences* **100**, 4150-4155 (2003).

17. Roy-Chowdhury, J., Locker, J. & Roy-Chowdhury, N. Nuclear receptors orchestrate detoxification pathways. *Developmental cell* **4**, 607-608 (2003).
18. Shapiro, S.M. Definition of the clinical spectrum of kernicterus and bilirubin-induced neurologic dysfunction (BIND). *J Perinatol* **25**, 54-9 (2005).
19. Bosma, P.J. Inherited disorders of bilirubin metabolism. *J Hepatol* **38**, 107-17 (2003).
20. Crigler, J.F., Jr. & Najjar, V.A. Congenital familial nonhemolytic jaundice with kernicterus. *Pediatrics* **10**, 169-80 (1952).
21. Arias, I.M., Gartner, L.M., Cohen, M., Ezzer, J.B. & Levi, A.J. Chronic nonhemolytic unconjugated hyperbilirubinemia with glucuronyl transferase deficiency. Clinical, biochemical, pharmacologic and genetic evidence for heterogeneity. *Am J Med* **47**, 395-409 (1969).
22. Berk, P.D., Wolkoff, A.W. & Berlin, N.I. Inborn errors of bilirubin metabolism. *Med Clin North Am* **59**, 803-16 (1975).
23. Seppen, J. et al. Discrimination between Crigler-Najjar type I and II by expression of mutant bilirubin uridine diphosphate-glucuronosyltransferase. *The Journal of clinical investigation* **94**, 2385-2391 (1994).
24. van der Veere, C.N. et al. Current therapy for Crigler-Najjar syndrome type 1: report of a world registry. *Hepatology* **24**, 311-5 (1996).
25. Chowdhury, J., Wolkoff, A., Chowdhury, N. & Arias, I. Hereditary jaundice and disorders of bilirubin metabolism. *The Metabolic and Molecular Bases of Inherited Disease. New York: McGraw-Hill* **2**, 3063-3101 (2001).
26. Hafkamp, A.M., Nelisse-Haak, R., Sinaasappel, M., Oude Elferink, R.P. & Verkade, H.J. Orlistat treatment of unconjugated hyperbilirubinemia in Crigler-Najjar disease: a randomized controlled trial. *Pediatr Res* **62**, 725-30 (2007).
27. Prasad, R., Rathi, R. & Kumar, P. 5. Biochemistry, molecular biology and molecular genetics of hyperbilirubinemia.
28. Tukey, R.H. & Strassburg, C.P. Genetic multiplicity of the human UDP-glucuronosyltransferases and regulation in the gastrointestinal tract. *Molecular Pharmacology* **59**, 405-414 (2001).
29. Sinaasappel, M. & Jansen, P.L. The differential diagnosis of Crigler-Najjar disease, types 1 and 2, by bile pigment analysis. *Gastroenterology* **100**, 783-9 (1991).
30. Sellier, A.L. et al. Successful plasmapheresis for acute and severe unconjugated hyperbilirubinemia in a child with crigler najjar type I syndrome. *JIMD Rep* **2**, 33-6 (2012).
31. Sticova, E. & Jirsa, M. New insights in bilirubin metabolism and their clinical implications. *World Journal of Gastroenterology: WJG* **19**, 6398 (2013).
32. Wolkoff, A.W. et al. Crigler-Najjar syndrome (type I) in an adult male. *Gastroenterology* **76**, 840-848 (1979).
33. Mitchell, E. et al. Hepatic parenchymal injury in Crigler-Najjar type I. *Journal of pediatric gastroenterology and nutrition* **66**, 588-594 (2018).
34. Fata, C.R., Gillis, L.A. & Pacheco, M.C. Liver fibrosis associated with Crigler–Najjar syndrome in a compound heterozygote: a case report. *Pediatric and Developmental Pathology* **20**, 522-525 (2017).
35. Gollan, J., Huang, S., Billing, B. & Sherlock, S. Prolonged survival in three brothers with severe type 2 Crigler-Najjar syndrome: ultrastructural and metabolic studies. *Gastroenterology* **68**, 1543-1555 (1975).
36. Fevery, J., Blanckaert, N. & Heirwegh, K.P. Unconjugated bilirubin and an increased proportion of bilirubin monoconjugates in the bile of patients with Gilbert's syndrome and Crigler-Najjar disease. *The Journal of clinical investigation* **60**, 970-979 (1977).
37. Ritter, J.K., Crawford, J.M. & Owens, I.S. Cloning of two human liver bilirubin UDP-glucuronosyltransferase cDNAs with expression in COS-1 cells. *J Biol Chem* **266**, 1043-7 (1991).
38. Ritter, J.K. et al. A novel complex locus UGT1 encodes human bilirubin, phenol, and other UDP-glucuronosyltransferase isozymes with identical carboxyl termini. *J Biol Chem* **267**, 3257-61 (1992).
39. Bosma, P.J. et al. Sequence of exons and the flanking regions of human bilirubin-UDP-glucuronosyltransferase gene complex and identification of a genetic mutation in a patient with Crigler-Najjar syndrome, type I. *Hepatology* **15**, 941-7 (1992).
40. Owens, I.S., Basu, N.K. & Banerjee, R. UDP-glucuronosyltransferases: gene structures of UGT1 and UGT2 families. *Methods Enzymol* **400**, 1-22 (2005).
41. Kadakol, A. et al. Genetic lesions of bilirubin uridine-diphosphoglucuronate glucuronosyltransferase (UGT1A1) causing Crigler-Najjar and Gilbert syndromes: correlation of genotype to phenotype. *Hum Mutat* **16**, 297-306 (2000).
42. Kaplan, M., Hammerman, C. & Maisels, M.J. Bilirubin genetics for the nongeneticist: hereditary defects of neonatal bilirubin conjugation. *Pediatrics* **111**, 886-93 (2003).
43. Montenegro Miranda, P.S. (Amsterdam, 2012).
44. Servedio, V. et al. Spectrum of UGT1A1 mutations in Crigler-Najjar (CN) syndrome patients: identification of twelve novel alleles and genotype-phenotype correlation. *Hum Mutat* **25**, 325 (2005).

45. Kadakol, A. et al. Genetic lesions of bilirubin uridine-diphosphoglucuronate glucuronosyltransferase (UGT1A1) causing Crigler-Najjar and Gilbert syndromes: Correlation of genotype to phenotype. *Human mutation* **16**, 297-306 (2000).
46. Strauss, K.A. et al. Management of hyperbilirubinemia and prevention of kernicterus in 20 patients with Crigler-Najjar disease. *Eur J Pediatr* **165**, 306-19 (2006).
47. Van der Veere, C.N. et al. Oral calcium phosphate: a new therapy for Crigler-Najjar disease? *Gastroenterology* **112**, 455-462 (1997).
48. Kappas, A., Drummond, G.S., Manola, T., Petmezaki, S. & Valaes, T. Sn-protoporphyrin use in the management of hyperbilirubinemia in term newborns with direct Coombs-positive ABO incompatibility. *Pediatrics* **81**, 485-497 (1988).
49. Galbraith, R.A., Drummond, G.S. & Kappas, A. Suppression of bilirubin production in the Crigler-Najjar type I syndrome: studies with the heme oxygenase inhibitor tin-mesoporphyrin. *Pediatrics* **89**, 175-182 (1992).
50. Sleisenger, M. Nonhemolytic unconjugated hyperbilirubinemia with hepatic glucuronyl transferase deficiency: a genetic study in four generations. *Transactions of the Association of American Physicians* **80**, 259-266 (1967).
51. Cremer, R.J., Perryman, P. & Richards, D. Influence of light on the hyperbilirubinaemia of infants. *The Lancet* **271**, 1094-1097 (1958).
52. Itoh, S. & Onishi, S. Kinetic study of the photochemical changes of (ZZ)-bilirubin IX α bound to human serum albumin. Demonstration of (EZ)-bilirubin IX α as an intermediate in photochemical changes from (ZZ)-bilirubin IX α to (EZ)-cyclobilirubin IX α. *Biochemical Journal* **226**, 251-258 (1985).
53. Zenone, E.A., Stoll, M.S. & Ostrow, J.D. The effect of elimination of environmental light on the metabolism of unconjugated bilirubin in the Gunn rat. *Dig Dis Sci* **27**, 1117-20 (1982).
54. Ennever, J.F. Blue light, green light, white light, more light: treatment of neonatal jaundice. *Clinics in perinatology* **17**, 467-481 (1990).
55. Stoll, M., Zenone, E. & Ostrow, J. Excretion of administered and endogenous photobilirubins in the bile of the jaundice gunn rat. *The Journal of Clinical Investigation* **68**, 134-141 (1981).
56. Tu, Z.-H. et al. Liver transplantation in Crigler-Najjar syndrome type I disease. *Hepatobiliary & Pancreatic Diseases International* **11**, 545-548 (2012).
57. Ostrow, J.D. Bile pigments and jaundice: Molecular, Metabolic, and medical aspects (Marcel Dekker Inc, 1986).
58. Dhawan, A. et al. Disease burden of Crigler–Najjar syndrome: Systematic review and future perspectives. *Journal of Gastroenterology and Hepatology* **35**, 530-543 (2020).
59. Yohannan, M.D., Terry, H.J. & Littlewood, J.M. Long term phototherapy in Crigler-Najjar syndrome. *Arch Dis Child* **58**, 460-2 (1983).
60. Lund, H. & Jacobsen, J. Influence of phototherapy on the biliary bilirubin excretion pattern in newborn infants with hyperbilirubinemia. *The Journal of pediatrics* **85**, 262-267 (1974).
61. Pett, S. & Mowat, A.P. Crigler-Najjar syndrome types I and II. Clinical experience--King's College Hospital 1972-1978. Phenobarbitone, phototherapy and liver transplantation. *Mol Aspects Med* **9**, 473-82 (1987).
62. Lysy, P.A. et al. Liver cell transplantation for Crigler-Najjar syndrome type I: update and perspectives. *World J Gastroenterol* **14**, 3464-70 (2008).
63. Burlina, A. & D'Antiga, L. in Pediatric Hepatology and Liver Transplantation 603-624 (Springer, 2019).
64. Schauer, R. et al. Treatment of Crigler-Najjar type 1 disease: relevance of early liver transplantation. *J Pediatr Surg* **38**, 1227-31 (2003).
65. Mentha, G. et al. [Results of a combined adult-child liver transplantation in Switzerland]. *Schweiz Med Wochenschr* **129**, 433-40 (1999).
66. Piselli, P. et al. Risk of de novo cancers after transplantation: results from a cohort of 7217 kidney transplant recipients, Italy 1997–2009. *European Journal of Cancer* **49**, 336-344 (2013).
67. Herrero, J.I. De novo malignancies following liver transplantation: impact and recommendations. (2009).
68. Sokal, E.M. et al. Orthotopic liver transplantation for Crigler-Najjar type I disease in six children. *Transplantation* **60**, 1095-1098 (1995).
69. Polgar, Z. et al. in Hepatocyte Transplantation 131-147 (Springer, 2017).
70. Ambrosino, G. et al. Isolated hepatocyte transplantation for Crigler-Najjar syndrome type 1. *Cell transplantation* **14**, 151-157 (2005).
71. Fox, I.J. et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* **338**, 1422-6 (1998).
72. Zhou, H. et al. Single liver lobe repopulation with wildtype hepatocytes using regional hepatic irradiation cures jaundice in Gunn rats. *PloS one* **7**, e46775 (2012).

73. Yannam, G.R. et al. A nonhuman primate model of human radiation-induced venocclusive liver disease and hepatocyte injury. *International Journal of Radiation Oncology* Biology* Physics* **88**, 404-411 (2014).
74. Soltys, K.A. et al. Host conditioning and rejection monitoring in hepatocyte transplantation in humans. *Journal of hepatology* **66**, 987-1000 (2017).
75. Chen, Y. et al. Amelioration of hyperbilirubinemia in gunn rats after transplantation of human induced pluripotent stem cell-derived hepatocytes. *Stem cell reports* **5**, 22-30 (2015).
76. Famulari, E.S. et al. Human liver stem cells express UGT1A1 and improve phenotype of immunocompromised Crigler Najjar syndrome type I mice. *Scientific reports* **10**, 1-14 (2020).
77. Fourrier, A. et al. Regenerative cell therapy for the treatment of hyperbilirubinemic Gunn rats with fresh and frozen human induced pluripotent stem cells-derived hepatic stem cells. *Xenotransplantation* **27**, e12544 (2020).
78. Strauss, K.A. et al. Crigler-Najjar Syndrome Type 1: Pathophysiology, Natural History, and Therapeutic Frontier. *Hepatology* **71**, 1923-1939 (2020).
79. Nathwani, A.C. et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* **371**, 1994-2004 (2014).
80. Gunn, C.K. Hereditary Acholuric Jaundice in the Rat. *Can Med Assoc J* **50**, 230-7 (1944).
81. Gunn, C. Hereditary acholuric jaundice: in a new mutant strain of rats. *Journal of Heredity* **29**, 137-139 (1938).
82. Lathe, G. An enzymatic defect in human neonatal jaundice and in Gunn's strain of jaundiced rats. *Biochem. J.* **67**, 9P (1957).
83. Roy-Chowdhury, N., Kondapalli, R. & Roy-Chowdhury, J. in Animal Models in Liver Research 149-171 (Academic Press New York, 1993).
84. Bortolussi, G. et al. Rescue of bilirubin-induced neonatal lethality in a mouse model of Crigler-Najjar syndrome type I by AAV9-mediated gene transfer. *FASEB J* **26**, 1052-63 (2012).
85. Nguyen, N. et al. Disruption of the ugt1 locus in mice resembles human Crigler-Najjar type I disease. *Journal of Biological Chemistry* **283**, 7901-7911 (2008).
86. Montenegro Miranda, P.S. & Bosma, P.J. Towards liver-directed gene therapy for Crigler-Najjar syndrome. *Current gene therapy* **9**, 72-82 (2009).
87. Aronson, S.J., Beuers, U. & Bosma, P.J. Progress and challenges in gene therapy for Crigler–Najjar syndrome. *Expert Opinion on Orphan Drugs* **3**, 1387-1396 (2015).
88. van Dijk, R., Beuers, U. & Bosma, P.J. Gene replacement therapy for genetic hepatocellular jaundice. *Clinical Reviews in Allergy & Immunology* **48**, 243-253 (2015).
89. Bortolussi, G. & Muro, A.F. Advances in understanding disease mechanisms and potential treatments for Crigler–Najjar syndrome. *Expert Opinion on Orphan Drugs* **6**, 425-439 (2018).
90. Ronzitti, G. et al. A translationally optimized AAV-UGT1A1 vector drives safe and long-lasting correction of Crigler-Najjar syndrome. *Mol Ther Methods Clin Dev* **3**, 16049 (2016).
91. Collaud, F. et al. Preclinical Development of an AAV8-hUGT1A1 Vector for the Treatment of Crigler-Najjar Syndrome. *Mol Ther Methods Clin Dev* **12**, 157-174 (2019).

Chapter 7

Preclinical development of an AAV8-hUGT1A1 vector for the treatment of Crigler-Najjar syndrome

Modified from
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Abstract

Adeno-associated viruses (AAV) are among the most efficient vectors for liver gene therapy. Results obtained in the first hemophilia clinical trials demonstrated the long-term efficacy of this approach in humans, showing efficient targeting of hepatocytes with both self-complementary (sc) and single-stranded (ss) AAV vectors. However, to support clinical development of AAV-based gene therapies, efficient and scalable production processes are needed. In an effort to translate to the clinic an approach of AAV-mediated liver gene transfer to treat Crigler-Najjar (CN) syndrome, we developed a (ss)AAV8 vector carrying the human UDP-glucuronosyltransferase- family 1-member A1 (hUGT1A1) transgene under the control of a liver-specific promoter. We compared our construct with similar (sc)AAV8 vectors expressing hUGT1A1, showing comparable potency in vitro and in vivo. Conversely, (ss)AAV8-hUGT1A1 vectors showed superior yields and product homogeneity compared with their sc counterpart. We then focused our efforts in the scale-up of a manufacturing process of the clinical product (ss)AAV8-hUGT1A1 based on the triple-transfection of human embryonic kidney (HEK) 293 cells grown in suspension. Large-scale production of this vector had characteristics identical to those of small-scale vectors produced in adherent cells. Preclinical studies in animal models of the disease and a good laboratory practice (GLP) toxicology/biodistribution study were also conducted using large scale preparations of vectors. These studies demonstrated long-term safety and efficacy of gene transfer with (ss)AAV8-hUGT1A1 in relevant animal models of the disease, thus supporting the clinical translation of this gene therapy approach for the treatment of CN syndrome.

1. Introduction

Crigler-Najjar (CN) syndrome is a rare and severe condition that affects bilirubin metabolism.[1, 2] The disease is due to mutations in the liver-specific enzyme uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) that conjugates bilirubin and mediates its disposal. The lack of this enzyme results in the accumulation of unconjugated bilirubin in serum, which is associated with brain toxicity and early death.[3] CN syndrome is an inherited autosomal recessive disorder with an incidence of about 1 in 1 000 000 newborns. Therapy for CN syndrome consists of daily cycles of 12-16 hours of exposure to blue light, which results in bilirubin photoisomerization and elimination in the urine. However, poor compliance to phototherapy and loss of efficacy associated with aging, expose patients to potentially lethal bilirubin spikes. [3, 4] At present, there is no authorized medicinal product for treatment of CN syndrome and orthotopic liver transplantation is the only curative treatment, with all the limitations of the approach. [3-6]

The monogenic etiology of the disease, the absence of liver parenchymal damage, and the low levels of UGT1A1 enzyme activity needed to correct the phenotype,[7] make CN syndrome an ideal target for gene therapy.[8, 9] Adeno-associated virus (AAV) vector-mediated gene therapy is one of the most promising approaches for safe and efficient gene transfer to the liver.[10] The efficacy of liver-directed AAV gene therapy is demonstrated by the recent successes in patients suffering from hemophilia A and B[11-15] and by the growing number of clinical trials using AAV vectors to transfer therapeutic transgenes to the liver.

Several studies showed therapeutic efficacy following AAV vector mediated gene transfer to the liver in both mouse and rat models of CN syndrome.[16-20] Accordingly, we recently showed long-term correction of bilirubin levels following liver-directed gene transfer with an AAV8 vector expressing a codon-optimized version of the UGT1A1 cDNA.[21]

However, one of the biggest challenges for the clinical translation of AAV vector-based therapies is vector production. In particular, scalable production systems able to deliver high-yield and high-purity material are needed.[22] Several AAV vector production platforms have been used to produce clinical-grade vector lots, although in recent years the field coalesced around transfection- and baculovirus- based production methods.[23]

Here, in an effort to develop and translate to the clinic a gene therapy for CN syndrome, we developed an AAV8 vector to efficiently express the human UGT1A1 transgene in hepatocytes. We initially compared single-stranded (ss) and self-complementary (sc) AAV vector genome configurations, showing a nearly identical potency profile *in vitro* and *in vivo*. However, based on the yields in production and the impurities profile, we focused production scale up and preclinical studies on a single-stranded AAV8-hUGT1A1 vector. A bioreactor system for the triple transfection human embryonic kidney (HEK)293 cells grown in suspension was established. Dose finding studies were conducted in two animal models of the CN syndrome using full-scale vector preparations. In parallel, good laboratory practice (GLP) studies were performed to evaluate toxicity, vector shedding, and biodistribution of the clinical vector (ss)AAV8-hUGT1A1 in wild-type rats. Germline transmission studies were also performed, together with a detailed assessment of immunogenicity of the therapeutic product. These results support the initiation of a phase I/II clinical trial in patients (NCT03466463).

2. Materials and methods

2.1. Test Items

Plasmids construct

The transgene expression cassette of the (ss)AAV8-hUGT1A1 carrying a codon-optimized cDNA sequence encoding for human UGT1A1 was previously described.[21] This construct (also known as GNT0003) was selected as the clinical candidate (NCT03466463). The cassette contains the human hemoglobin beta (HBB)-derived synthetic intron (HBB2)[24] and the HBB polyadenylation signal. The transgene expression cassette of the (sc)AAV8-hUGT1A1 was developed by Dr Bosma (Academic Medical Center, Amsterdam, Netherlands) and consisted of a codon-optimized cDNA sequence encoding for human UGT1A1 transgene cloned into a (sc)AAV backbone. This plasmid carries the AAV2 inverted terminal repeats (ITR)s with an intact 5'terminal resolution site (trs) without the analogous 3'trs, a hybrid liver-specific promoter (HLP), a modified SV40 small intron sequence[25] and the SV40 late polyadenylation signal. For the in vitro comparison of hUGT1A1 expression levels, we used a (ss)AAV8-GFP as a control. The expression cassette contained the cDNA sequence encoding for enhanced green florescent protein (eGFP) transgene, the phosphoglycerate kinase (PGK) promoter, and a SV40 polyA sequence. For the in vivo comparative study between mice and rats, we used a (ss)AAV8 vector expressing coagulation factor IX under the transcriptional control of a liver specific promoter.[26]

Production of AAV vectors

Research-grade AAV vectors used in this study were produced using a slight modification of the adenovirus-free transient transfection methods described.[23] Briefly, adherent HEK293 cells grown in roller bottles were transfected with the three plasmids containing the adenovirus helper proteins, the AAV Rep and Cap genes, and the ITR-flanked transgene

expression cassette. 72 hours after transfection, cells were harvested, lysed by sonication, and treated with Benzonase® (Merck-Millipore, Darmstadt, Germany). Vectors were then purified using two successive ultracentrifugation rounds in cesium chloride density gradients. Full capsids were collected, the final product was formulated in sterile phosphate buffered saline containing 0.001% of Pluronic (Sigma Aldrich, Saint Louis, MO), and stored at -80 °C. GMP-like (ss)AAV8-hUGT1A1 vectors used in this study were produced in bioreactor and at different scales up to 200 liters by adenovirus-free transient transfection method. Suspension HEK293 cells were transfected with Polyethyleneimine (PEI) (PEIpro, Polyplus) with the three same plasmids mentioned above. Twenty-four hours after transfection, cells were treated with Benzonase® and two days later, they were lysed with Triton ((Sigma, St Louis, MO) and clarified by filtration. Vectors were purified by a single chromatography column based on AVB Sepharose immuno-affinity (GE Healthcare) before concentration by tangential flow filtration. Purified particles were formulated in Ringer-Lactate solution containing 0.001% Pluronic (F68), vialed and stored at −80 °C.

Characterization of AAV vectors

Titers of AAV vector stocks were determined using quantitative real-time PCR (qPCR). Specific probe and primers were as follows:

forward 5'-GGCGGGCGACTCAGATC-3',
reverse 5'-GGGAGGCTGCTGGTGAATATT-3',
probe 5'-AGCCCCTGTTTGCTCCTCCGATAACTG-3'

To perform alkaline agarose gel, viral DNA were extracted from 100µL of AAV solutions. Samples were denatured 5 minutes at 95°C and prepared in 6X alkaline loading buffer (300mM NaOH, 6mM EDTA, 18% Ficoll, 0.5% bromocresol green, 0.25% xylene cyanol) and GelRed1X. After electrophoresis in denaturing conditions (50mM NaOH-1mM EDTA), gel was stained with GelRed diluted 1:10 000.

Analytical Ultra Centrifugation measures the sedimentation coefficient of macromolecules by following over time the optical density of a sample subjected to ultracentrifugation. The difference in the sedimentation coefficient, measured by Raleigh interference or 260 nm absorbance, depends on the content of viral genome in the capsid. AUC analysis was performed using a Proteome Lab XL-I (Beckman Coulter, Indianapolis, IN). 400µL of AAV vector and 400µL of formulation buffer were loaded into a two-sector velocity cell. Sedimentation velocity centrifugation was performed at 20,000 rpm and 20°C. Absorbance (260 nm) and Raleigh interference optics were used to simultaneously record the radial concentration as a function of time until the lightest sedimenting component cleared the optical window (approximately one hour and a half). Absorbance data required the use of extinction coefficients to calculate the molar concentration and the percent value of the empty and genome-containing capsids. Molar concentrations of both genome-containing and empty capsid were calculated using Beer's law and % full genome-containing and empty capsid was calculated.

For the characterization of the VP proteins content in the different populations of particles, AAV8-hUGT1A1 vectors were loaded in cesium chloride solution (1.38g/cm^3) and ultra-centrifuged at 38000rpm and 20°C for 40h. The total volume of the viral/cesium solution was then collected in 16 fractions (0.5mL). The CsCl was removed from the fractions by repeated dialysis cycles against PBS-0.001% pluronic F68 solution. A volume of 10µL of each fraction were loaded onto a 4-12% SDS-polyacrylamide gel (Thermo Fisher Scientific,

Waltham, MA). After protein migration, the gel was stained with a SYPRO® Ruby solution (Invitrogen, Carlsbad, CA).

2.2 *In vitro* experiments

hUGT1A1 expression in Huh7 cell line

For transduction experiments, six-well plates containing 80% confluent Huh-7 cells were transduced with AAV8-UGT1A1 vectors at the indicated MOI. AAV8-GFP transduced cells were included as control. 72 hours after transduction cells were harvested and frozen at -20°C until processing. Microsomes extraction was performed at 4°C. Frozen cell pellets were resuspended in 300µL of lysis buffer (20mM Hepes, 1% Triton X-100), proteases inhibitor cocktail (Sigma Aldrich, Saint Louis, MO) and centrifuged 5 minutes at 100xg. Supernatants were collected and centrifuged 60 minutes at 18000 g. Pellets were resuspended in 100µL of 20mM Hepes and the protein concentration was determined using the BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's instructions. Microsomal extracts were separated on a 4-12% bis-tris polyacrylamide gel (Thermo Fisher Scientific, Waltham, MA). The same amount of protein was loaded in each lane. The gel was transferred onto a nitrocellulose membrane and blotted with an anti-UGT1A1 antibody (SantaCruz biotechnology, Santa Cruz, CA) and an anti-actin antibody (Sigma Aldrich, Saint Louis, MO), used as loading control. Secondary antibodies and detection system were from Li-Cor Biosciences (Lincoln, NE).

Phenobarbital experiment

Six-well plates containing 80% confluent Huh-7 cells were exposed to various phenobarbital concentrations (0, 40, 200, 500 and 1000µM). 24 hours after, cells were transduced with (ss)AAV8-UGT1A1 vectors at the indicated MOI. 72 hours after transduction cells were harvested and pellets were frozen at -20°C until processing. Microsomes extraction and Western-Blot were performed as described above. Total RNA were prepared using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. 1 µg of total RNA was reverse-transcribed using RevertAid H Minus First Strand cDNA Synthesis Kit ((Thermo Scientific, Waltham, MA) and oligodT primer according to manufacturer's instructions. Total cDNA (1µl) was used to perform Real Time (RT)-qPCR using specific primers :

UGT1A1 forward 5'-GGCGGGCGACTCAGATC-3'
reverse 5'-GGGAGGCTGCTGGTGAATATT-3'
GAPDH forward 5'- CGAACACCATGGGGAAGGTGAAG -3'
reverse 5'- CGTGTGACCAGGCGCCCAATA -3'

RT-qPCR data were analyzed using the ΔΔCt method.

2.3 *In vivo* studies

Ugt1$^{-/-}$ mice have been described previously.[16] Wild type or Ugt1$^{-/-}$ untreated littermates were used as controls as indicated. Mice were housed and handled according to institutional guidelines, and experimental procedures approved by the International Centre for Genetic Engineering and Biotechnology board. Animals used in this study were at least 99.8 %

C57BL/6 genetic background, obtained after more than 9 backcrosses with wild type C57BL/6 mice. Mice were kept in a temperature-controlled environment with 12/12 hour light–dark cycle. They received a standard chow diet and water ad libitum.

The Gunn rat is a natural occurring model of CN syndrome that has no residual UGT1A1 enzyme activity. Rats and wild-type C57BL/6 mice used in this study were fed ad libitum and were housed and handled according to institutional guidelines. All in vivo experimental procedures were approved by the French, and Italian competent authorities and Ethical Committees (ref. 2013007C and 2017002B_APAFIS#9667) according to the European Directive 2010/63/EU.

Both male and female animals were used, to better represent the CN patient population which includes both male and female individuals.

Gene transfer procedures and phototherapy treatment

Ugt1$^{-/-}$ mice

Post-natal day 11 (P11) mice were intraperitoneally injected with AAV vectors or saline. Newborns were exposed to blue fluorescent light (Philips, Amsterdam, The Netherlands) for 12hrs per day (synchronized with the light period of the light/dark cycle) up to 12 days after birth and then maintained under normal light conditions. Intensity of the blue lamps was monitored monthly with an Olympic Mark II Bili-Meter (Olympic Medical, Port Angeles, WA). For adult studies, Ugt1$^{-/-}$ animals were exposed to blue fluorescent lamps up to day 20 after birth as previously described.[20] Blood samples were collected at sacrifice by facial vein exsanguination. Liver tissues were collected at sacrifice for transgene expression assessment. For immunogenicity study and glucocorticoids administration evaluation, PBS or AAV vectors were administrated in adult (P60-P90) Ugt1$^{-/-}$ mice by intravenous (i.v) injection in retro-orbital sinus. Blood samples and liver tissues were collected at sacrifice for transgene expression assessment.

Gunn rats

6-8 weeks old rats were injected in i.v via the tail vein with AAV vectors or saline. Blood samples were collected by retro-orbital venipuncture every week after AAV injection. To determine transgene expression, liver tissues were collected at sacrifice. For analysis of bilirubin glucuronides, bile samples were collected in this experiment at 3 months post injection.

C57BL6 mice

6-8 weeks old mice were injected in i.v. via the tail vein with AAV vectors or saline. Blood samples were collected by retro-orbital venipuncture and liver tissues were collected at sacrifice.

Bilirubin measurement

Total Bilirubin determination in mice and rats was performed in plasma as previously described. Plates were read at 560 nm on an Enspire plate reader (Perkin Elmer, Waltham, MA). Bilirubin conjugates in bile were analyzed and quantified by HPLC as previously

described[18] using an Omnisphere column (Varian, Palo Alto, CA) for the separation of bilirubin conjugates.[27]

hFIX measurement

Levels of hFIX in plasma were quantified with an enzyme-linked immunosorbent assay (ELISA) as described.[26] Capture antibody (PierceTM Factor IX Antibody - MA1-43012) was used at 2.8µg/mL, left overnight at 4°C, washed with PBS containing 0.05% Tween 20 (PBST), and blocked with 200µL/well of blocking buffer (6% milk in PBST) for 2 hours at 37°C. Standards were prepared by serial dilutions of purified recombinant hFIX (starting concentration, 1000µg/mL). A horseradish peroxidase (HRP)–conjugated goat polyclonal anti-hFIX Ab (Affinity Biologicals, Ancaster, ON, Canada) diluted 1:1000 was used as secondary antibody.

Transaminases measurement

Activities of ALT and AST in plasma were measured using colorimetric ALT and AST activity assay kits (Sigma, St Louis, MO). Plates were read on an Enspire® plate reader (Perkin Elmer, Waltham, MA).

Virus vector genome copy number analysis

To reduce variability generated by uneven transduction of liver parenchyma by AAV vectors, whole rat livers were homogenized in 20mM Hepes, 250mM sucrose. For mouse samples, livers were harvested 1 month post-injection, pulverized in liquid nitrogen and aliquoted for further molecular analysis. The following primers and probes were used :

UGT1A1 forward 5'-GGCGGGCGACTCAGATC-3'
reverse 5'-GGGAGGCTGCTGGTGAATATT-3'
probe 5'-AGCCCCTGTTTGCTCCTCCGATAACTG-3'

TITIN forward 5'-AGAGGTAGTATTGAAAACGAGCGG-3'
reverse 5'-GCTAGCGCTCCCGCTGCTGAAGCTG-3'
probe 5'-TGCAAGGAAGCTTCTCGTCTCAGTC-3'

VGCN in mice was quantified by qPCR using specific primers for the hAAT promoter as previously described.[17]

Preparation of total RNA and RT-PCR analysis

Total RNA from mouse liver was prepared using EuroGOLD Trifast (Euroclone) according to manufacturer's instructions. 1 µg of total RNA was reverse-transcribed using M-MLV (Invitrogen, Carlsbad, CA) and oligodT primer according to manufacturer's instructions. Total cDNA (1µl) was used to perform either RT-PCR or qPCR using specific primers :

forward 5'-TAAATACGGACGAGGACAGG-3'
reverse 5'-ACCTCCTTGTGATTCCACAG-3'

qPCR was performed using iQTM SYBR® Green Supermix (Bio-Rad, Hercules, CA) and a C1000 Thermal Cycler CFX96 Real Time System (Bio-Rad, Hercules, CA). Expression of the gene of interest was normalized to Gapdh house-keeping gene, using specific primers :

forward 5'-ATGGTGAAGGTCGGTGTGAA-3'
reverse 5'- GTTGATGGCAACAATCTCCA-3'

Real Time PCR data were analyzed using the ΔΔCt method.

Microsomes extraction and Western blot analysis

From 500µL of liver homogenate, microsomes extraction was performed at 4°C. Rat liver samples were centrifuged 5min at 100g, mixed with 4.5mL of 20mM Hepes buffer and centrifuged at 10000g for 10min. Supernatants were then centrifuged at 20000g for 60min. Pellets were resuspended in 400µL of 20mM Hepes and the protein concentration was determined using the BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's instructions. Microsomal extracts were separated on a 4-12% bis-tris polyacrylamide gel (Thermo Fisher Scientific, Waltham, MA). The same amount of protein was loaded in each lane. The gel was transferred onto a nitrocellulose membrane and blotted with an anti UGT1 rabbit polyclonal antibody (SantaCruz Biotechnology, Santa Cruz, CA). Anti-actin (Sigma Aldrich, Saint Louis, MO), was used as loading control. Secondary antibodies and detection system were from LiCor Biosciences (Lincoln, NE).

For mouse samples, liver powder was homogenized in RIPA Buffer (150mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50mM Tris HCl pH8, 2x protease inhibitors) and analyzed by Western blot analysis as described previously.[16]

Methylprednisolone treatment

Gunn rats

6-8 week old rats were divided into four groups. Animals were treated either with i.v. (ss)AAV8-hUGT1A1 vector or PBS on day 0. Later the rats were treated i.p. daily with Methylprednisolone (SoluMedrol) or PBS utilizing the following schedule: 5mg/kg prednisolone for 7 days, 2.5mg/kg for next 2 days, 1.3mg/kg for next 2 days, 0.6mg/kg for next 2 days and 0.2mg/kg for last 2 days. The groups that received PBS on Day 0 were treated with prednisolone or PBS between days 21 and 35. The groups that received rAAV8-hUGT1A1 vector on day 0 were treated with prednisolone or PBS between days 28 and 42. Blood samples were collected during three months after AAV injection at several time points. Animals were sacrificed on day 84 and liver were collected.

Ugt1$^{-/-}$ mice

Adult mice (8-12 weeks old) were injected retro-orbitally with (ss)AAV8-hUGT1A1 at the dose of $1x10^{12}$ or $5x10^{12}$ vg/kg. One day before the vector treatment animals were treated i.p. with methylprednisolone (Solu-Medrol). Methylprednisolone was applied as five consecutive daily injections at the doses of 5.0, 2.5, 1.3, 0.6, 0.2mg/kg/day. Animals were sacrificed on day 84 and blood and liver were collected.

Rapamycin treatment

Eight week old Gunn rats were divided into two groups. Both groups were treated intravenously (i.v.) with (ss)AAV8-hUGT1A1 vector on day 0. Immunomodulation with rapamycin injected i.p. at 1.5mg/kg/day was started 2 days prior to vector infusion and continued for 16 days. Animals were followed for 3 months and blood samples were collected at several time points. Animals were sacrificed on day 85 and liver were collected.

GLP toxicity/biodistribution study

This study was performed in Charles River (Tranent, UK) and Genosafe (Evry, France) laboratories. Hundred 5-7 old wild-type Sprague Dawley rats were divided in 3 groups. Animals were treated either with (ss)AAV8-hUGT1A1 vector at $5x10^{12}$ or at $2.5x10^{13}$ vg/kg or with PBS on day 0 by intravenous injection into the lateral tail vein. Follow up of 3 weeks, 3 months and 6 months were realized on the animals to evaluate the potential toxicity of the vector.

Clinical signs, body weight changes, food and water consumption, and clinical pathology parameters were evaluated during this study. For hematology, coagulation, clinical chemistry, urinalysis and immunogenicity assessment, blood and urines samples were collected in each group at different time points from day 6 to the day of sacrifice. Urines and blood samples were also collected for shedding analysis at days 2, 4, 6, 15 and 28 after injection. Gross necropsy findings, organ weights, biodistribution of the vector and histopathologic examinations were conducted in animals euthanized 8, 91 and 182 days after vector administration.

GLP germline transmission study

This study was performed in Centre de Recherche Biologiques (CERB) (Baugy, France) and Genosafe (Evry, France) laboratories. Adult New Zealand White male rabbits were used for this study. (ss)AAV8-hUGT1A1 vectors or vehicle were administered once on day 1 by the intravenous route as a slow bolus over about 1 minute in the marginal ear vein in a volume of 0.9mL/kg. Morbidity/mortality checks were performed twice daily. Clinical observations were performed before dosing and daily during the study. Body weights were recorded on day 1, 3, 5, 7, 9, 15 and then once a week. Sperm samples were collected before dosing and on day 3, 7, 15, 30, 60, 90, 120 and 150. All animals were sacrificed on day 150. Selected organs (testis and epididymis, liver) were sampled on the day of necropsy then analyzed for biodistribution assessment.

GLP In Situ Hybridization (ISH)

This study was performed by Cytoxlab (Evreux, France). Briefly, the ISH method was automated and performed on Formalin-Fixed Paraffin-Embedded (FFPE) tissues. The sections were pre-treated by heating at +97°C for 24min and subsequently incubated with a protease for another 16min at 37°C. The probes of interest (Advanced Cell Diagnostic Inc., Newark, CA) were then hybridized at +43°C for 2hrs. The amplification and detection systems were applied following the manufacturer recommendations (RNAscope® 2.5 VS, Advanced Cell Diagnostic Inc., Newark, CA). The sections were stained with hematoxylin for 8min and subsequently bluing reagent for 4min. The slides were thoroughly washed in

soap water for 2min followed by running water for additional 2min. Finally, all slides were mounted.

Detection of Anti-AAV8 Antibodies in Rat Plasma

MaxiSorp 96-wells plates (Thermo Fisher Scientific, Waltham, MA) were coated with AAV8 capsids in carbonate buffer at 4°C overnight. A standard curve of rat IgG (Sigma Aldrich, Saint Louis, MO) prepared as seven 2-fold dilution steps starting at 1µg/mL was coated onto the wells. After blocking, plasma samples were added to plates and incubated 1 hour at 37°C. Secondary antibody was added into well (anti-Rat IgG-HRP) and plates were developed with 3,3′,5,5′-tetramethylbenzidine substrate and the optical density was assessed by spectrophotometry at 450 and 570nm (for background subtraction) on Enspire plate reader (Perkin Elmer, Waltham, MA) after blocking the reaction with 5% H2SO4.

Detection of Anti-UGT1A1 Antibodies in Rat Plasma

Human UGT1A1

Same ELISA protocol as described above was followed, but using for this specific experiment, the UGT1A1 protein to coat the plates.

Rat UGT1A1

To detect antibodies against the rat UGT1A1 protein, 40µg of microsomal extracts from liver tissues expressing or not rat UGT1A1 transgene were separated on a 4-12% bis-tris polyacrylamide gel (Thermo Fisher Scientific, Waltham, MA) and blotted onto a nitrocellulose membrane. Serum of each rat was used to develop a separate membrane as primary antibody (1:100). As a positive control, serum from rats immunized with UGT1A1 (1:200) and commercial anti-UGT1 rabbit polyclonal antibody (Millipore, Burlington, MA) were used. A positive signal, corresponding to the rUGT1A1 band, is detected only when antibodies against rUgt1a1 are present in the plasma of the animal. Secondary antibodies and detection system were from Li-Cor Biosciences (Lincoln, NE).

Detection of Anti-UGT1A1 Antibodies in Mouse Plasma

The determination of anti-human Ugt1a1 antibodies in mouse plasma has been described previously,[19, 20] while the determination of anti-mouse UGT1A1 antibodies was performed with a minor modification of the previous protocol. HuH7 cell line was infected with an AAV8 containing the mouse WT UGT1A1 cDNA (25000 MOI) and 72hrs later cell were harvested and total cell protein extract was prepared. 20µg of total protein extract derived from uninfected and AAV-infected cells were separated in a 10% sodium dodecacyl sulfate polyacrylamidegel electrophoresis, blotted onto nitrocellulose membrane. Plasma from individual animals was used as primary antibody (1:200).

Histological analysis of liver sections

At sacrifice livers biopsies from AAV-treated animals and wild-type and Ugt1$^{-/-}$ untreated littermates were extracted and fixed with 4% paraformaldehyde (PFA) in PBS overnight at

4°C. The next day biopsies were immersed in 20% sucrose in PBS and 0.02% sodium azide and kept at 4°C.

For immunofluorescence analysis and Oil red staining after cryoprotection in specimens were frozen in optimal cutting temperature compound (BioOptica, Milano, Italy) and 14µm slices were obtained in a cryostat. For Ugt1a1 immunofluorescence liver specimens (14µm) were incubated in sodium citrate pH9 prior to blocking solution. Next, specimens where blocked in 10% normal goat serum (Dako) and then incubated with the primary antibody (1:200; Sigma, St. Louis, MO) for 2hrs at room temperature. Specimens were incubated with secondary antibody Alexa fluor 488, Invitrogen). Nuclei were visualized by adding Hoechst (10µg/ml) and mounted with Mowiol 4-88 (Sigma). Oil red staining was performed according to manufacturer instructions (Bioptica, Milano, Italy). Periodic-acid staining (Sigma, St. Louis, MO) was performed according to manufacturer instructions. Sirius red staining, sections were de-waxed and hydrated; nuclei were stained with Weigert hematoxylin, washed in tap water and then incubated with picro-sirius red for 1 hour (Sigma, St. Louis, MO). After two washes in acidified water sections were dehydrated in absolute ethanol, cleared in xylene and mounted in Eukitt (Sigma, St. Louis, MO). Images were acquired on a Nikon Eclipse18 E-800 epi-fluorescent microscope with a charge-coupled device camera (DMX 1200F; Nikon,19 Amstelveen, The Netherlands). Digital images were collected using ACT-1 (Nikon) software.

Integration Sites analysis

This study was performed by Genwerk GmbH (Heidelberg, Germany). Liver samples from six adult $Ugt^{-/-}$ mice (3 females and 3 males) treated with a single intravenous injection of (ss)AAV8-hUGT1A1 at $5x10^{12}$ vg/kg and two pools (male and female) of controls mice were analyzed. Liver were collected 9 months after AAV administration.

LAM-PCR, nrLAM-PCR, library preparation and sequencing

LAM-PCR (4-fold analysis, each restriction enzyme used in duplicate) was used to identify AAV vector flanking genomic sequences in DNA isolated from liver samples. The detailed protocol has been previously described.[28] In this study, 500 ng of genomic DNA were used with MluCI or CviQI as restriction enzymes. Briefly, regions adjacent to the AAV ITR are initially amplified in two consecutive 50-cycles linear PCR steps employing a single biotinylated primer hybridizing upstream of the vector's 5-prime ITR. To allow the later amplification of the unknown sequence, purified biotinylated PCR products are digested to enable the cohesive end ligation of a double-stranded linker cassette carrying a sample-specific barcode. Barcoded fragments are then amplified in two nested exponential PCRs using biotinylated vector- and adaptor-specific primers**.**

nrLAM-PCR was performed in duplicate on DNA isolated from liver samples to identify the genomic sequences flanking the AAV vector avoiding the restriction bias introduced in the LAM-PCR due to the use of restriction enzymes. The detailed protocol has been previously described.[29] To enable amplification of the unknown sequence, a single stranded linker cassette carrying a sample-specific barcode is ligated to the biotinylated PCR products purified by magnetic capture. Barcoded fragments are then amplified in two nested exponential PCRs using biotinylated vector- and adaptor-specific primers.

LAM-PCR and nrLAM-PCR amplicons were prepared for high throughput sequencing by an Illumina MiSeq platform as previously described[29, 30], employing fusion-primers carrying

MiSeq-specific sequencing adaptors in the second nested exponential PCR, as well as a sample-specific barcode to enable parallel sequencing of multiple samples in a single sequencing run. Libraries were visualized by electrophoresis on the Agilent 2200 TapeStation system for QC and quantification purposes. (nr)LAM-PCR libraries were sequenced in the Illumina MiSeq platform at the German Cancer Research Center Sequencing Facility by 450+50-nucleotide paired-end sequencing using Illumina 500-cycle kit.

Bioinformatical analysis

Sequence reads were parsed with the aim of detecting signs of vector fragments in any orientation. Only vector fragments with a length of 20 bases or more are used as rearrangements. The structure of the blocks is reconstructed on any single read in order to capture the topology of the rearrangement. The recurrence rate of each rearrangement containing R=1,2,…,7 blocks (rearrangement order) is then taken into account in order to retrieve the block topology.[31] Sequencing technologies like Illumina MiSeq allow for a semi-quantitative estimation of clonal size by the counting of the number of retrieved sequences (retrieval frequency) for individual vector-genome junctions (IS). The relative sequence count of all detected IS was calculated in relation to all sequences which could be mapped to a definite position in the genome. The ten most prominent IS (Rank 1: highest sequence count; Rank 2: 2nd highest sequence count; Rank 10: 10th highest sequence count) were analyzed for each sample. The detailed analysis using our in-house pipeline GENE-IS (current version 1.6) has been previous described[31, 32] Genomic regions enriched in integration events, termed common integration sites (CIS) may permit to detect domains potentially involved in the perturbation of relevant cellular processes. To identify CIS, we employ a computational framework based on graphs[33] visualized in Cytoscape version 2.8.3.[34]

Statistical analysis

Results are expressed as mean ± SEM or mean ± SD as described in the text. The Prism package (Graph Pad Software, La Jolla, CA) or StatistiXL plugin for windows Excel were used to analyze data and prepare the graphs. Statistical analysis has been performed by one way or two-way ANOVA as described in the text. Values of $p< 0.05$ were considered as statistically significant.

3. Results

3.1 AAV genome configuration design

(ss)AAV8-hUGT1A1 vector preparations present better yields and higher product homogeneity compared to (sc)AAV8-hUGT1A1 vectors

We compared (sc)AAV and (ss)AAV vectors encoding for hUGT1A1 under the transcriptional control of liver specific promoters (**Figure 7.1A**). The two expression cassettes were pseudotyped into AAV serotype 8 to efficiently transduce the liver of animals.[12, 35] Research grade vector preparations were obtained by triple transfection of adherent HEK293 cells grown in roller bottles.[23] Purification by cesium chloride gradient of

(ss)AAV8-hUGT1A1 resulted in two well-defined bands, corresponding to empty and full particles. Conversely, the full particle band obtained for (sc)AAV-hUGT1A1 was less intense and diffused (**Figure 7.1B**). This was associated with more than three-fold reduction in production yields (6.0±1.89 x10^4 vector genome (vg)/cell vs. 1.77±1.37 x10^4 vg/cell for (ss) and (sc)AAV, respectively, data obtained from at least two independent production lots).

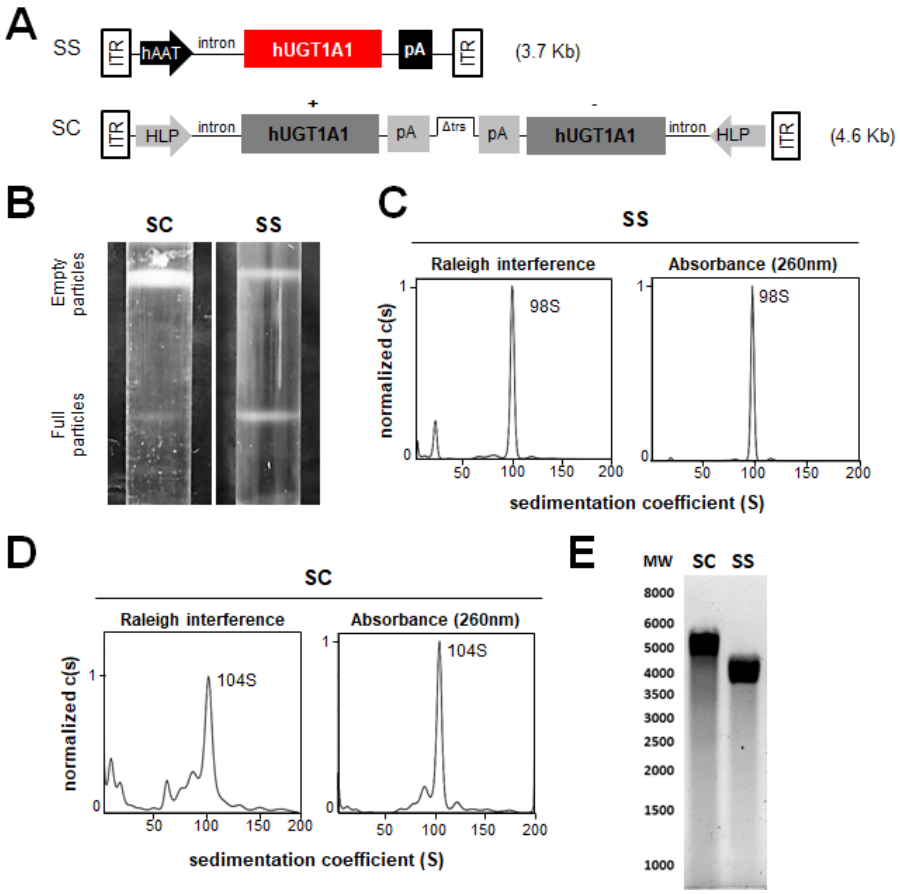

Figure 7.1 (sc)AAV8-hUGT1A1 and (ss)AAV8-hUGT1A1 vectors comparison.
(A) Schematic representation of the transgene expression cassettes. (ss)AAV8-hUGT1A1 (SS) and (sc)AAV8-hUGT1A1 (SC) cassettes are composed by a liver specific promoter, an intron, a poly-A (pA) and the sequence of human UGT1A1 (hUGT1A1). SC expression cassette contains a truncated ITR (Δtrs) that allows for self-complementary genome packaging. (B) Purification profiles of (ss)AAV8-hUGT1A1 (SS) and (sc)AAV8-hUGT1A1 (SC) vectors after ultracentrifugation in cesium chloride gradient. The higher bands contain empty particles and the lower bands contain full particles. (C-D) Sedimentation profiles of (ss)AAV8-hUGT1A1 (C) and (sc)AAV8-hUGT1A1 (D) full particles purified by cesium chloride gradient and subjected to analytical ultracentrifugation. In each graph, the X axis represents the sedimentation coefficient expressed in Svedberg units (S), and the Y axis represents the normalized value of the concentration as a function of S (c(S)) measured by Railegh interference or absorbance at 260 nm. (E) Denaturing gel performed on genomic extracts obtained from (sc)AAV8

We then evaluated the analytical ultracentrifugation (AUC) profile of the full particles obtained for the two vectors. AUC performed on purified (ss)AAV8-UGT1A1 vectors, revealed, a sharp, single peak, with a sedimentation coefficient consistent with the size of the single stranded genome (**Figure 7.1C**). Conversely, for (sc)AAV8-hUGT1A1 vectors we observed the presence of different peaks, possibly due to the presence of AAV vectors containing DNA fragments of variable length (**Figure 7.1D**). The integrity of the genome was further analyzed by separation of purified vector genomes by electrophoresis under alkaline condition (**Figure 7.1E**), confirming the presence of a smear of vector genomes of different sizes in (sc)AAV8-hUGT1A1 vector preparations. Together, these data indicate

that research grade (ss)AAV8-hUGT1A1 vectors were produced with higher yields and were more homogeneous than their self-complementary counterpart.

(ss) and (sc)AAV8-UGT1A1 vectors present equal potency profile *in vitro* and *in vivo*

We next compared the transduction efficiency of (sc)AAV8-hUGT1A1 vs. (ss)AAV8-hUGT1A1 *in vitro* in human hepatoma cells,[19] which resulted in a similar expression of the hUGT1A1 transgene (**Figure 7.2A**). Next, *in vivo* comparison of the vectors was performed in a well-established model of CN syndrome, the Gunn rat.[36-38] This model presents blood levels of bilirubin comparable to those observed in patients with mild symptoms and no evident signs of brain toxicity (6-12 ± mg/dL).

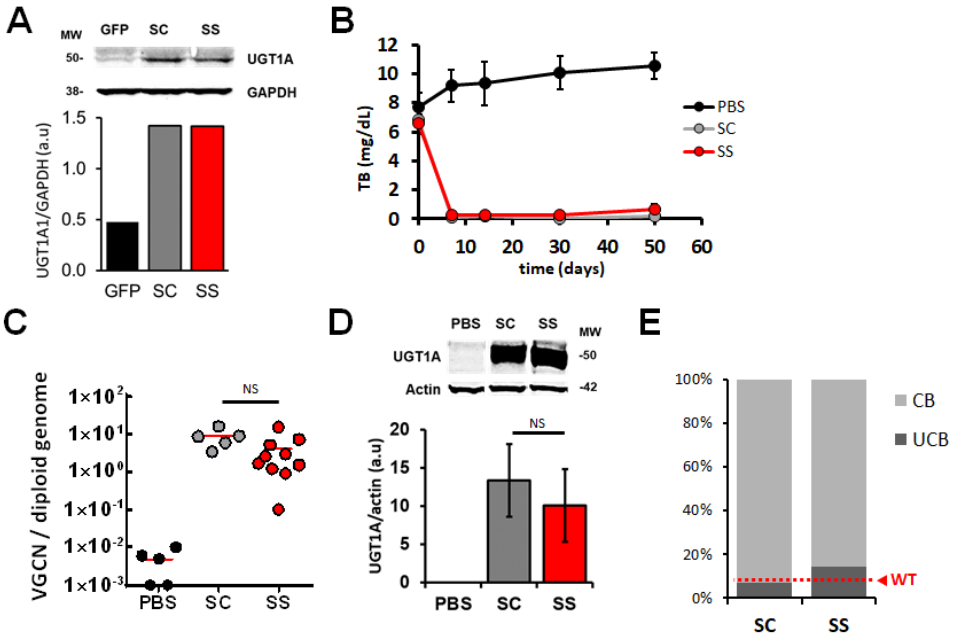

Figure 7.2 (sc)AAV8-hUGT1A1 and (ss)AAV8-hUGT1A1 vectors have similar efficacy in vitro and in vivo
(A) Huh7 cells were transduced with AAV8-GFP, (sc)AAV8-hUGT1A1 or (ss)AAV8-hUGT1A1 at 25000 multiplicity of infection. 72 hours after transduction, microsomal extracts were obtained, separated by SDS-PAGE and analyzed by western blot with UGT1A and GAPDH specific antibodies. Molecular weight (MW) is indicated on the left and quantification of band intensity is reported. (B-D) 6-8 week-old Gunn rats were injected with PBS (PBS), 1x10^{12}vg/rat of (sc)AAV8-hUGT1A1 (SC) or the same dose of (ss)AAV8-hUGT1A1 (SS). (B) Total bilirubin (TB) levels measured in serum at the indicated time points. (C) Vector genome copy number (VGCN) per cell measured by qPCR in liver. The graph shows the single values (dots) and the average values (red line) measured in each group and normalized for the number of copies of titin per sample. Statistical analysis was performed by ANOVA (NS, non-significant; PBS n=5, SC n=5, SS n=10). (D) Western blot analysis performed on microsomal extracts from rat livers with UGT1A and actin specific antibodies. Molecular weight (MW) is indicated on the left and quantification of band intensity is reported. (E) Bilirubin conjugates measured by HPLC in the bile of 8 week-old Gunn rats injected with 5x10^{11}vg/kg of (sc)AAV8-hUGT1A1 (SC, n=6) or (ss)AAV8-hUGT1A1 (SS, n=10). Twelve weeks after injection, animals were sacrificed, and bile was analyzed. The graph shows the percentage of conjugated bilirubin (CB) and unconjugated bilirubin (UCB) measured in bile. The level of unconjugated bilirubin observed in wild-type wistar rats is reported in red (WT).

(sc)AAV8-hUGT1A1 and (ss)AAV8-hUGT1A1 vectors titered side-by-side were injected intravenously in 8-week-old Gunn rats (n=10/group). Total bilirubin (TB) was measured in blood for about 7 weeks post-injection. Systemic injection of 1x10^{12} vg/rat (corresponding to 5.0x10^{12} vg/kg) of both vectors resulted in a complete and sustained normalization of circulating bilirubin levels (**Figure 7.2B**). Vector genome copy number (VGCN) analysis and hUGT1A1 protein expression levels in liver measured in treated animals confirmed the equivalence in potency of the two vectors (**Figure 7.2C-D**). A similar potency of (ss) vs. (sc) AAV8-hUGT1A1 vectors was also observed at 1x10^{10} and 1x10^{11} vg/rat (data not

shown). In a separate experiment, Gunn rats were injected with $5x10^{11}$ vg/kg of (ss)AAV8-hUGT1A1 or (sc)AAV8-hUGT1A1. Three months after vector injection, bilirubin-glucuronide conjugates were present at similar levels in the bile of rats treated with the two vectors (**Figure 7.2E**). These data suggest that (ss) and (sc)AAV8-hUGT1A1 vectors used in the current study transduce hepatocytes equally both in vitro and in vivo. Based on the comparable efficacy of the two vectors, and the higher production yields and homogeneity profile, the (ss)AAV8-hUGT1A1 vector was selected as lead candidate for clinical development

3.2 Manufacturing process development

A fully scalable method based on triple transfection of HEK293 cells cultured in suspension was developed to support the clinical development of (ss)AAV8-hUGT1A1 (**Figure 7.3**). Triple transfection of HEK293 cells was performed with polyethylenimine (PEI) directly in 10-liter bioreactors. AAV vectors were recovered from both supernatant and cells by mild detergent lysis followed by AVB Sepharose affinity column purification.

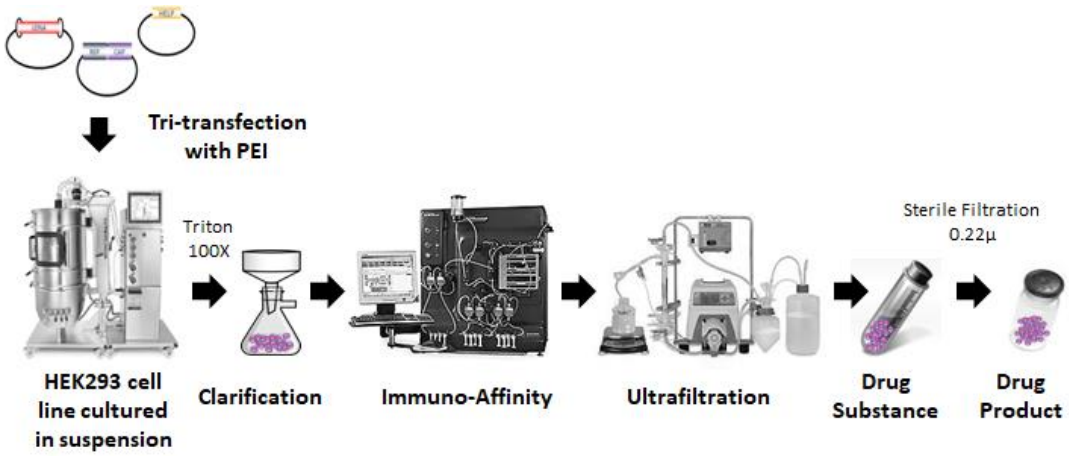

Figure 7.3 AAV large-scale manufacturing process scheme
HEK293 cells which were previously adapted to suspension culture are expanded to the desired volume in stirred tank bioreactors (2L, 10L or 200L). The cells are transiently transfected with 3 plasmids complexed to polyethylenimine (PEI) to generate AAV8-hUGT1A1 vectors. After the production phase, the cells are disrupted using Triton detergent treatment and the crude material is then clarified by filtration. The AAV capsids are purified by capture chromatography based on the immune-affinity matrix AVB Sepharose (GE Healthcare). The eluted vectors are subsequently concentrated by tangential flow filtration in hollow fibers and diafiltered with the formulation buffer (Ringer Lactate + 0.001% Pluronic F68). After sterile filtration on a 0.2µm filter, the final product is vialed and stored at -80°C.

Purified vectors were then concentrated and tested for quality and potency. (AUC) performed on (ss)AAV8-hUGT1A1 vector produced in suspension revealed that the product contained around 20% of full capsids (data not shown). To better characterize the full to empty particles ratio, the purified (ss)AAV8-hUGT1A1 final product was loaded on a cesium chloride gradient and 16 fractions were collected and analyzed for capsid protein and DNA content. SDS-PAGE followed by Sypro® Ruby staining revealed the presence of the three bands corresponding to VP1, VP2 and VP3 at a ratio of 1:1:10 (**Figure 7.4A**). Two populations of particles, peaking at fraction 8 and 12-13 were identified. Subsequent quantitative PCR (qPCR) analysis showed that fraction 8 and 12-13 corresponded to full and empty particles, respectively (**Figure 7.4B**). Consistent with the AUC analysis, AAV particles present in fraction 8 represented 21% of the total VP protein, and qPCR data indicated that this fraction contained full capsids, representing 72.9% of AAV genomes

present in all fractions. We then evaluated the *in vitro* potency of the vector produced in suspension. Huh-7 cells were transduced with increasing multiplicity of infections (MOIs) of (ss)AAV8-hUGT1A1 produced in adherent cells or in suspension at 10L scale. Similar levels of hUGT1A1 protein were detectable by Western blot in cell lysates obtained from cells transduced with the two vector preparations (**Figure 7.4C**).

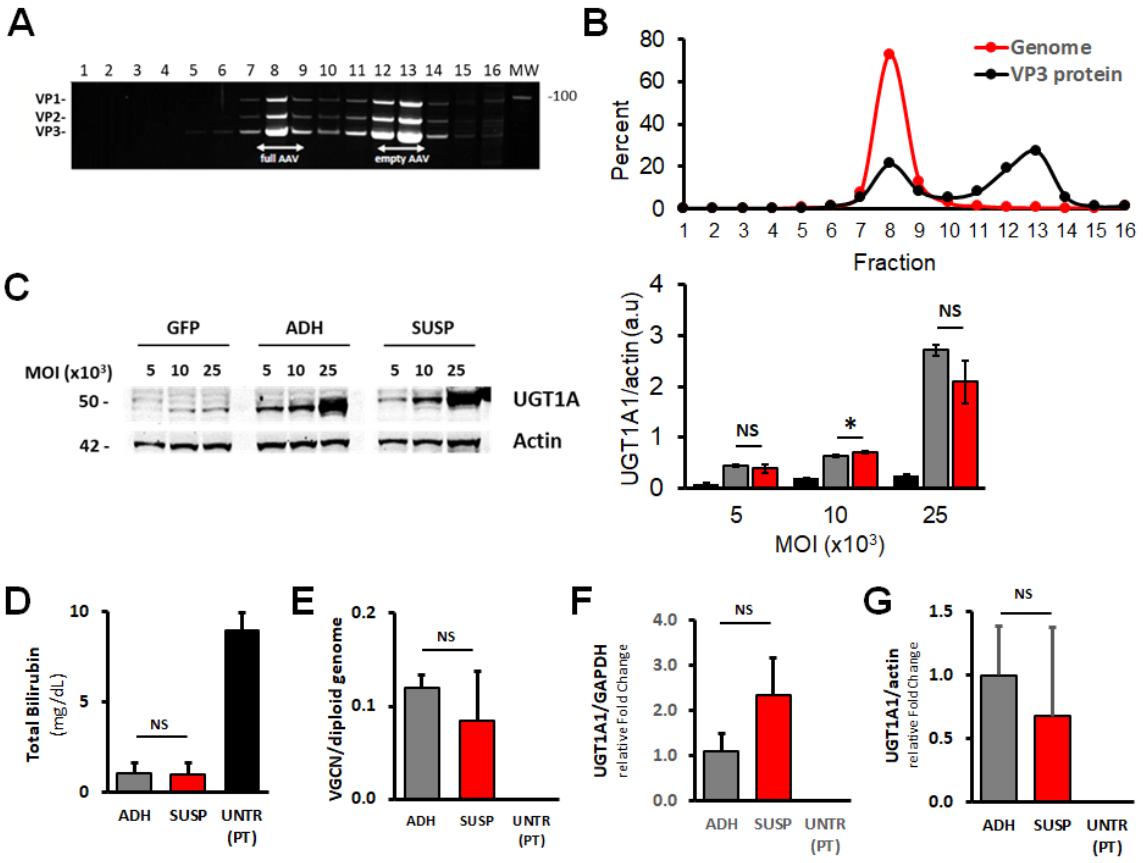

Figure 7.4 In vitro and in vivo comparison of (ss)AAV8-hUGT1A1 produced in HEK293 cells growing in suspension or adherent.
(A-B) Characterization of AAV8 vector expressing hUGT1A1 produced by triple transfection of HEK293 cells cultured in suspension in medium scale bioreactor (10L) and purified by affinity chromatography (SUSP). (A) Suspension-produced vector was loaded in a cesium chloride gradient ultracentrifugated and fractionated in 16 fractions starting from the bottom of the tube. Each fraction was analyzed by SDS-PAGE (lanes 1 to 16) and stained with SYPRO Ruby. Molecular weight is indicated on the right. (B) VP3 intensity of each fraction was quantified and the percentage of each fraction was plotted in the graph (VP3 protein). Genome titer was measured in each fraction by qPCR. The percentage was calculated as the number of copies in each fraction divided by the sum of the copies in the whole fractions (Genome) (C). Huh7 cells were transduced with AAV8-GFP (GFP), (ss)AAV8-hUGT1A1 produced in HEK293 cells cultured in adhesion (ADH) or in suspension (SUSP) at 5000, 10000 and 25000 multiplicity of infection (MOI). 72 hours post-transduction, microsomal extracts were obtained, separated by SDS-PAGE and analyzed by western blot with UGT1A and actin antibodies. Molecular weight is indicated on the left. Quantification of band intensity is reported on the right. (D-G) In vivo comparison in a mouse model of hyperbilirubinemia. Ugt1a1$^{-/-}$ mice were injected with vehicle (PBS) or 3.3x10^{9}vg/mouse of (ss)AAV8-hUGT1A1 vectors produced in HEK293 cells in adhesion (ADH) or in suspension (SUSP). (D) TB levels analyzed 30 days after vector injection. (E) Vector genome copy number (VGCN) analysis, (F) normalized hUGT1A1 mRNA levels and (G) protein expression by Western blot in livers of Ugt1a1-/- mice analyzed 30 days post treatment. Data are reported as mean ± standard deviation. Statistical analyses were performed by ANOVA (=p<0.05; NS, non-significant; UNTR (PT) n=4, ADH n=4, SUSP n=3).*

The equivalence of the two vectors was further confirmed *in vivo* in a juvenile Ugt1 knock-out mouse model (Ugt1$^{-/-}$).[16, 17] These mice present a phenotype that closely resembles the human condition, therefore they need to be exposed to phototherapy (PT) to survive. Eleven-day-old mice were injected with 3.3x10^{9} vg/mouse (corresponding to 5.0x10^{11} vg/kg) of (ss)AAV8-hUGT1A1 vector produced in adherent or in suspension HEK293 cells. One-

month post injection plasma bilirubin levels were determined and a complete correction of total serum bilirubin levels (about 1.0 mg/dl) was observed in mice treated with both AAV vector preparations (**Figure 7.4D**). VGCN analysis demonstrated that the two vectors transduced hepatocytes with similar efficacy (**Figure 7.4E**). Accordingly, expression of hUGT1A1 mRNA and protein levels in the livers of AAV vectors-treated Ugt1$^{-/-}$ mice analyzed 30 days post-treatment was comparable (**Figure 7.4F-G**). These results demonstrate that potency of vectors produced in bioreactors are fully comparable with research grade vectors derived from adherent cells.

We next scaled-up the 10-liter suspension AAV production to 200 liters. Several 200-liter lots of (ss)AAV8-hUGT1A1 vectors were produced, demonstrating robustness and reproducibility of the process. The vector potency was then evaluated *in vitro*. Treatment of Huh-7 cells at a 25,000 MOI of (ss)AAV8-hUGT1A1 resulted in hUGT1A1 protein levels similar to those obtained with adherent cells and medium scale (**Figure 7.5**). AUC analysis revealed that the large-scale product contained >30% of full capsids (**Figure 7.6A**).

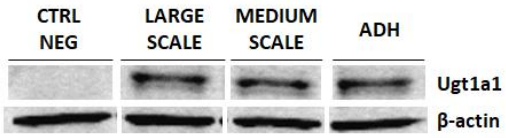

Figure 7.5 In vitro potency of (ss)AAV8-hUGT1A1 produced in HEK293 cells growing in suspension at large scale. Huh7 cells were transduced with AAV8-GFP (CTRL NEG), (ss)AAV8-hUGT1A1 produced in HEK293 cells cultured in adhesion (ADH) or in suspension at 50L (MEDIUM SCALE) or 200L scale (LARGE SCALE). Multiplicity of infection of 25000 was used. 72 hours post-transduction, microsomal extracts were obtained, separated by SDS-PAGE and analyzed by Western Blot with UGT1A and actin antibodies.

3.3 Primary pharmacodynamics: dose-finding studies

Next, a dose escalation study was conducted in Gunn rats, starting at vector doses that have previously shown therapeutic efficacy.[19] Two weeks after systemic injection of 6-8 week-old rats, blood samples were collected, and total bilirubin was analyzed. A dose-dependent decrease in levels of plasma TB was seen in animals treated with the (ss)AAV8-hUGT1A1 (**Figure 7.6B**). At a dose of $1x10^{12}$ vg/kg, 4/11 rats showed correction of TB levels, while at $2x10^{12}$ vg/kg in 8/10 rats a full correction of hyperbilirubinemia was observed (**Figure 7.6B**, and data not shown). Vector genome copies determined in whole livers of treated Gunn rats 84 days after treatment also showed a dose-dependent increase and correlated with the levels of correction of TB in serum. (**Figure 7.6C**). Based on these results, VGCN below 0.1 copies/cell were not sufficient to correct TB levels, as 13/14 animals with VGCN < 0.1 copies/cell had TB levels > 5 mg/dL. VGCN/cell in the range of 0.1 to 0.6 mediated TB correction in 6/14 rats, while all animals with VGCN/cell greater than 0.6 had normal TB levels.

Dose finding studies were also performed in Ugt1$^{-/-}$ mice. Adult (60-90 days old) male and female Ugt1$^{-/-}$ mice were injected with different doses of (ss)AAV8-hUGT1A1 (from $1.0x10^{11}$ to $5.0x10^{12}$ vg/kg) (**Figure 7.7**). Plasma bilirubin levels were monitored for 9 months post-injection. Male mice showed bilirubin levels below 0.5mg/dl at all the doses tested, starting at $1x10^{11}$ vg/kg (**Figure 7.7A**). In female animals, in contrast, after the initial drop of TB levels to WT levels one month post-injection (**Figure 7.7B**), some animals lost expression over time (see **Figure 7.8** for single animal TB levels).

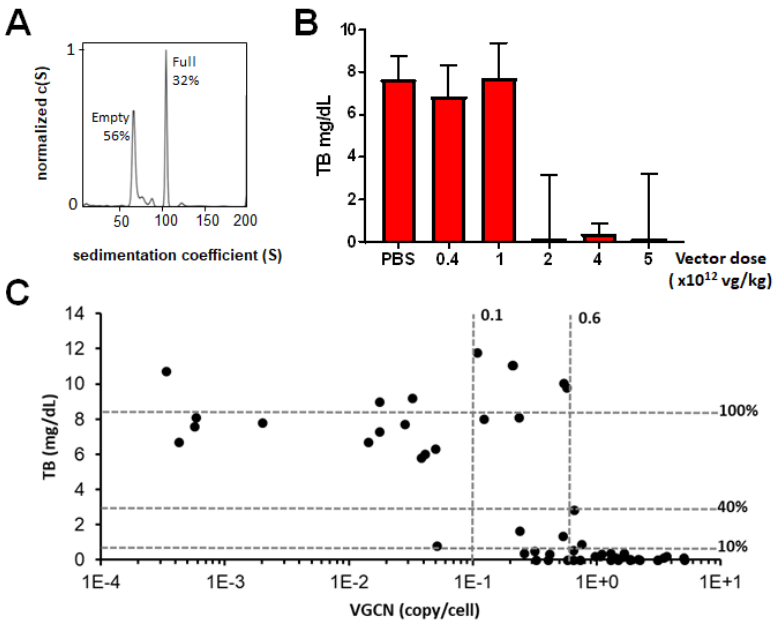

Figure 7.6 Non-GLP dose-finding study with large scale-produced (ss)AAV8-hUGT1A1 vector.
(A) Sedimentation profile obtained by analytical ultracentrifugation of (ss)AAV8-hUGT1A1 vector, produced by triple transfection of HEK293 cells in suspension at large scale (150L) and purified by affinity chromatography. The X axis represents the sedimentation coefficient expressed in Svedberg units (S), and the Y axis represents the normalized value of the concentration as a function of S (c(S)) measured by Raleigh interference. (B-C) 8 week-old Gunn rats were injected i.v. with PBS (PBS) or (ss)AAV8-hUGT1A1 at indicated doses. Total bilirubin (TB) levels in plasma and vector copy number (VGCN) in liver were assessed 84 days post-injection. (B) Median with range of TB levels in plasma are shown. (C) TB levels plotted against VGCN in liver. Data from all animals are shown

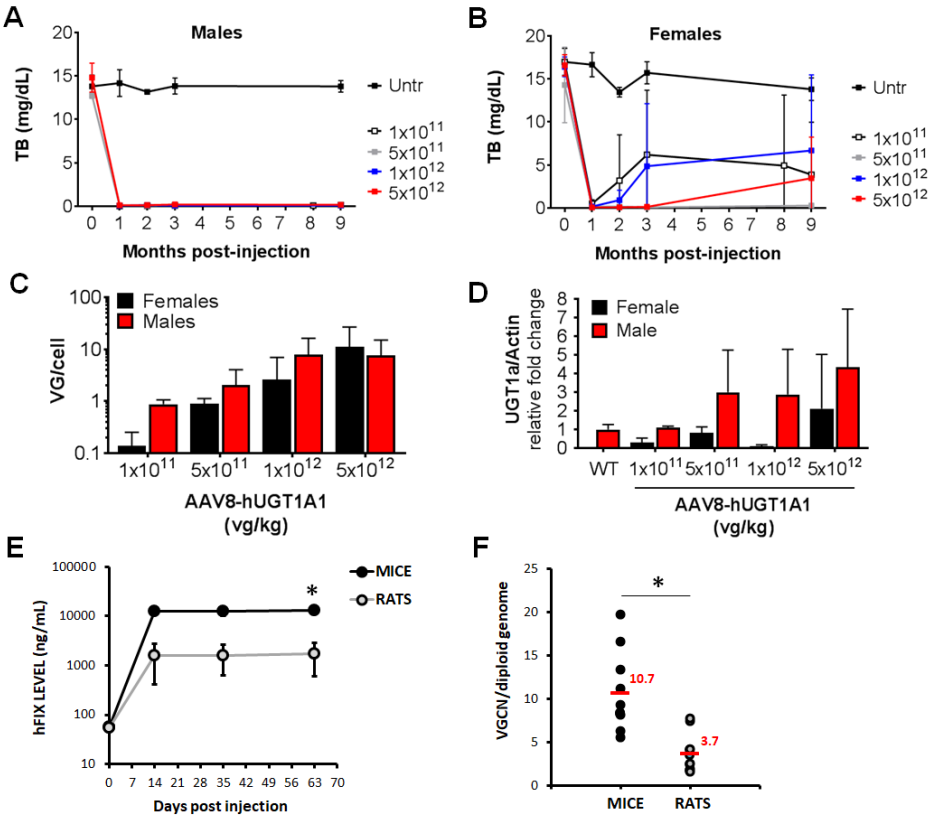

Figure 7.7 Non-GLP dose-finding study of (ss)AAV8-hUGT1A1 vector in adult Ugt1$^{-/-}$ mice.
Ugt1$^{-/-}$ mice were injected as adults with the indicated doses (vg/kg). At 1, 2 and 3 months post-injection mice were bled and TB levels in plasma were analyzed. Nine months post injection mice were sacrificed and molecular analysis were carried out. Plasma bilirubin determination in male (A) and female (B) Ugt1$^{-/-}$ mice. (C) Viral genome copy number

(VG/cell) at 9 months post injection in male and female mice treated with different doses of rAAV8-hUGT1A1. (D) Western blot analysis of liver total protein extracts at 9 months post injection. 1x10^{11} dose male n=5 and female n=5; 5x10^{11} dose male n=3 and female n=2; 1x10^{12} dose male n=8 and female n=5; 5x10^{12} dose male n=6 and female n=5. (E, F) Wistar rats (WT and UGT1A1 deficient rats; n=10) and C57Bl/6 mice (n=10) were injected with 5x10^{12} vg/kg of an AAV8 vector expressing human coagulation FIX (hFIX). (E) Levels of circulating hFIX were measured by ELISA and reported in the graph. (F). Liver genome copy number analysis performed 2 months after vector injection. Data are shown as genome copy number per cell normalized on titin gene. Statistical analyses were performed by ANOVA (=p<0.05 as indicated).*

Nine months post-injection mice were sacrificed, and livers were harvested for molecular analysis. VGCN (vg/cell) in whole livers showed a dose-response course (**Figure 7.7C**) and well correlated with the level of Ugt1a1 protein determined by Western blot analysis and immunofluorescence (**Figure 7.7D** and **Figure 7.9**). Some inconsistencies in vector dose response were noted in female animals, likely due to sex-specific differences in the efficiency of liver transduction in rodents.[39] Histological analysis of livers revealed no obvious abnormalities, as determined by H&E, Masson trichromic, Sirius red, PAS and Oil red-stainings (**Figure 7.10**).

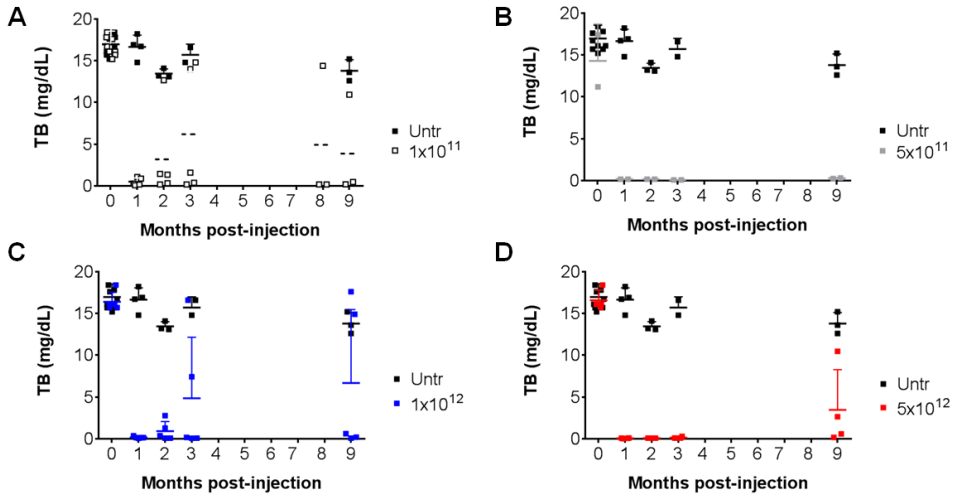

Figure 7.8 Plasma bilirubin in female Ugt1$^{-/-}$ mice.
Plasma bilirubin determination in female Ugt1$^{-/-}$ mice treated with 1x10^{11}vg/kg (A), 5x10^{11}vg/kg (B), 1x10^{12}vg/kg (C) and 5x10^{12}vg/kg (D). Each dot represents a single animal. Untr, untreated Ugt1$^{-/-}$ mice.

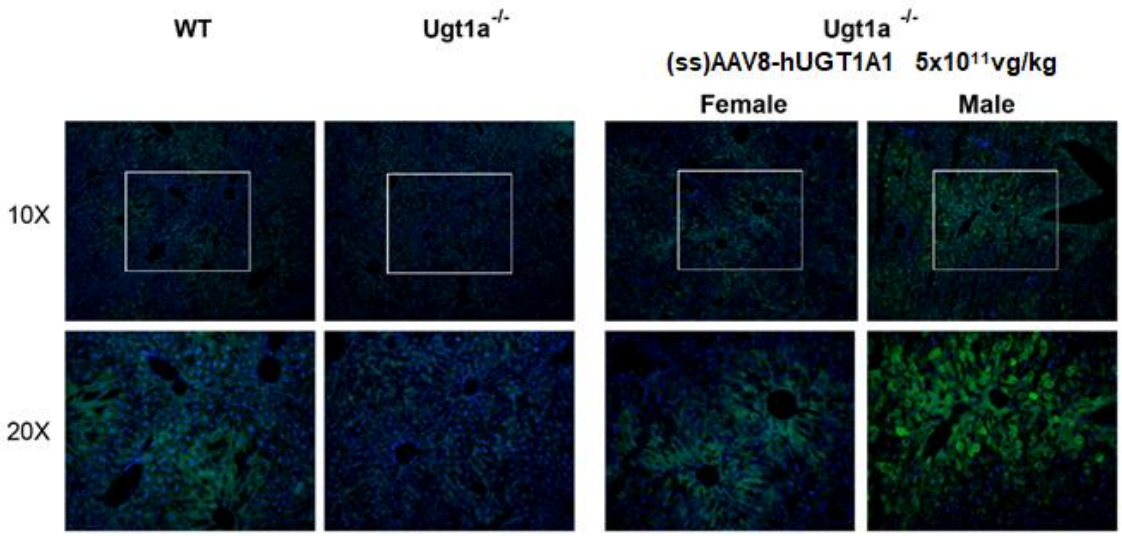

Figure 7.9 UGT1A1 expression in mice liver
Immunofluorescence analysis of liver sections from WT, Ugt1$^{-/-}$ untreated and treated with 5x10^{11}vg/kg vector dose. Rectangles show magnification images shown below. Results are expressed as mean ± SD.

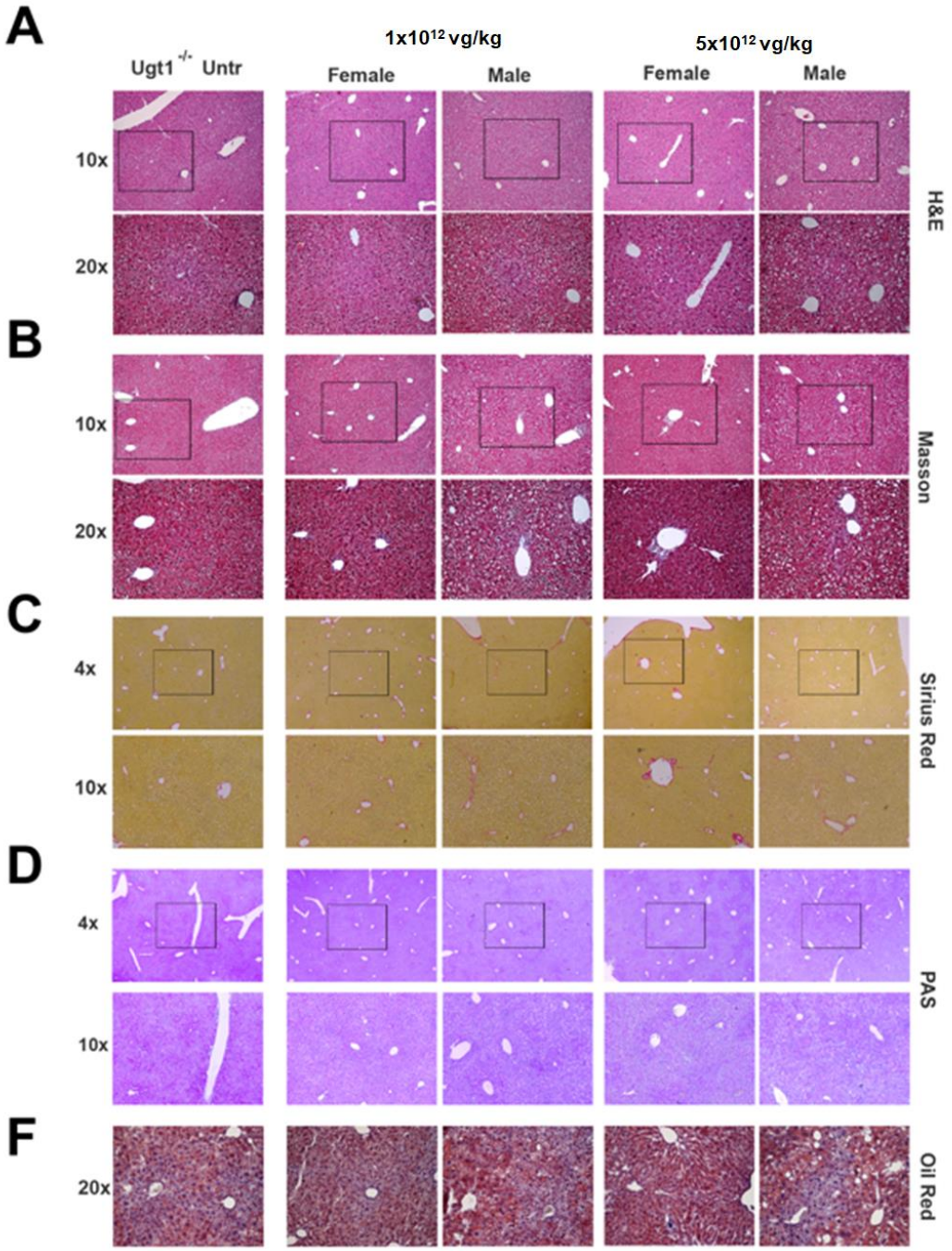

Figure 7.10 Histological analysis in Ugt1$^{-/-}$ treated animals.
(A) Representative images of H&E staining; (B) Masson trichrome; (C) Sirius red; (D) PAS staining and (F) Oil red. Ugt1$^{-/-}$ untreated animals where used as control. Rectangles show magnification images shown below.

We next performed an additional comparison study in mice and rats using an AAV8 expressing human coagulation factor IX (hFIX) under the transcriptional control of a liver specific promoter. We first ran a pilot study which demonstrated that the extent of liver transduction of AAV vectors in rats of Wistar and Sprague Dawley background is similar (data not shown). Then, we compared hFIX expression levels in Wistar rats (both wild-type and *Ugt1a1*$^{-/-}$) with C57Bl/6 mice. At a dose of 5x10^{12} vg/kg, mice showed levels of hFIX >10µg/mL (**Figure 7.7E**), while rats expressed approximately 1.5µg/mL of hFIX. Interestingly, hFIX expression levels in rats were in a similar range, although still higher, of those observed in hemophilia B patients receiving 2x10^{12} vg/kg of a similar vector.[12] The

evaluation of the vector genome copy number in the two species supported the findings on hFIX transgene expression levels (**Figure 7.7F**).

Together, these results indicate that the rat is a good predictive model of the efficacy of liver gene transfer in humans, in particular for AAV8 vectors, and support further preclinical studies including toxicology/biodistribution study in this species.

3.4 Pharmacodynamic interactions

Phenobarbital does not interfere with (ss)AAV8-hUGT1A1 gene transfer

To reduce the risk of kernicterus, CN I and some severe CN II patients need daily PT. Because they have a residual UGT1A1 enzyme activity, CN II patients are also conventionally treated with phenobarbital, that can enhance partially this enzyme activity.[40, 41] Recently, clinicians have noted that discontinuation of phenobarbital therapy may lead to elevated hepatic transaminases in these patients. High levels of transaminases unfortunately represent an exclusion criteria of a liver gene therapy trial. Also, in order to keep these patients eligible for the trial, they have to stay under phenobarbital.

Consequently, investigations were made to identify possible interactions of phenobarbital with (ss)AAV8-hUGT1A1 gene transfer. Specifically, the vector potency was evaluated *in vitro*, in presence of phenobarbital in the culture medium. Transduction of phenobarbital pretreated Huh-7 cells with (ss)AAV8-hUGT1A1 resulted in hUGT1A1 protein and mRNA levels similar to those obtained with cells cultured without phenobarbital (**Figure 7.11**).

These results indicate that no negative effects are expected on the (ss)AAV8-hUGT1A1 gene transfer efficacy in presence of phenobarbital.

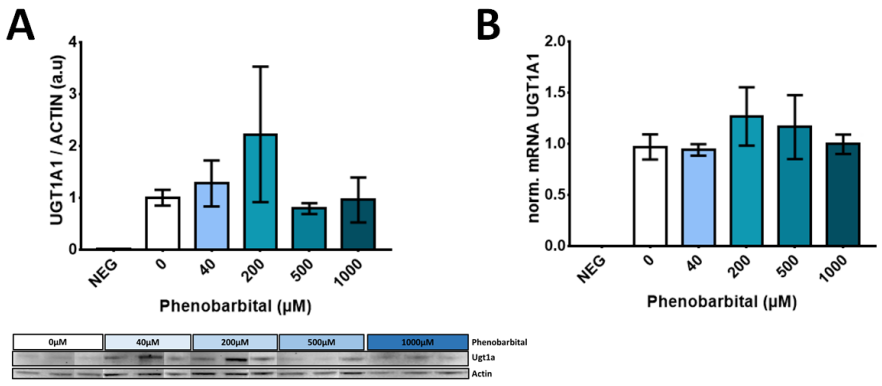

Figure 7.11 Phenobarbital has no detrimental effect on (ss)AAV8-hUGT1A1 transduction efficacy
Huh7 cells were exposed to various phenobarbital concentration for 24h (0, 40, 200, 500, or 1,000µM). Then, they were transduced with PBS (NEG) or (ss)AAV8-hUGT1A1 at 50.000 MOI. 72 hours post-transduction, hUGT1A1 protein (A) and mRNA (B) levels were analyzed. Data are expressed as mean ± SD of triplicates. hUGT1A1 protein levels were normalized on the level of actin and then to the levels observed in cells transfected without phenobarbital. UGT1A1 mRNA levels were normalized to the expression levels of GAPDH and then normalized for the levels measured in cells transfected without phenobarbital. Statistical analyses have been performed by ANOVA.

Administration of glucocorticoids in conjunction with (ss)AAV8-hUGT1A1 gene transfer is safe in mouse and rat models of Crigler-Najjar syndrome.

Immune response triggered by AAV is a potential concern for gene therapy clinical applications, and based on the experience with AAV8-mediated liver gene transfer for

hemophilia B, it is known that a short course of corticosteroids may be needed to control the immune response directed against the vector.[11, 12] To identify possible interactions of corticosteroids with AAV gene transfer in CN animals, we used both Gunn rats and Ugt1$^{-/-}$ mice. In Gunn rats, a tapering course of corticosteroid was given 4 weeks post gene transfer. No detrimental effect on correction of TB levels was observed (**Figure 7.12A**). Accordingly, no effect of methylprednisolone treatment on VGCN was noted (**Figure 7.12B**), and no obvious treatment-related effects on transaminase activities were measured (**Figure 7.13A**).

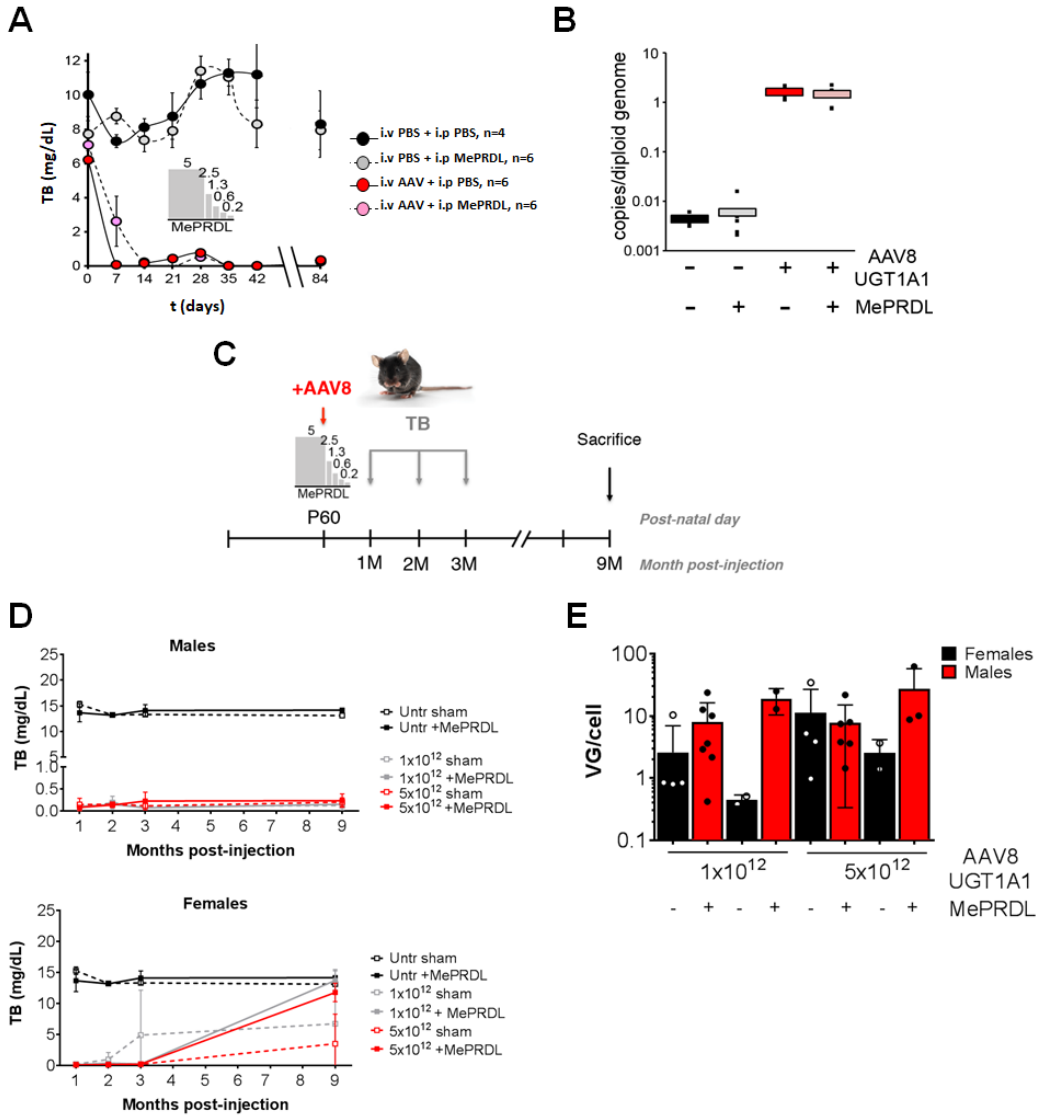

Figure 7.12 Corticosteroid treatment in CN animal models does not affect gene transfer efficacy.
(A-B) Gunn rats were injected with PBS (black) or 5x10^{12}vg/kg of (ss)AAV8-UGT1A1 (red) at day 0. One month after the treatment, a 15-day tapering dose of methyl-prednisolone was administered intraperitoneally in half of the rats (grey and pink). Rats were weekly bled and sacrificed 3 months after vector injection. (A) Total bilirubin (TB) levels were weekly measured and plotted versus time. Data are expressed as mean ± standard error. The internal bar-chart describes the tapering course of methyl-prednisolone (MePRDL). (B) Vector genome copy number (VGCN) per cell was measured by qPCR performed on genomic material extracted from livers of Gunn rats. Values were normalized for the number of copies of genomic DNA using titin as standard. In the figure are showed the single values measured (small black dots) and the median value for each treatment. (C-E) Ugt1$^{-/-}$ mice were injected as adults with the indicated doses (vg/kg). A MePRDL treatment was initiated the day before the injection of the AAV vector and lasted for 5 days at decreasing doses (5.0, 2.5, 1.3, 0.6 and 0.3 mg/kg). At 1, 2 and 3 months post-injection mice where bled and TB levels in plasma where analyzed. Nine months post injection mice where sacrificed and molecular analysis where carried out. (C) Experimental strategy. (D) TB levels in plasma in male and female Ugt1$^{-/-}$ mice after treatment with different doses (vg/kg) of rAAV8-mUGT1A1 with or without corticosteroids. (E) VGCN in male and female mice 9 months post injection. Results are expressed as mean ± SD and individual values are plotted. 1x10^{12} dose male sham n=7, male + MePRDL n=2, female sham n=5 and female + MePRDL n=2; 5x10^{12} dose male sham n=6, male + MePRDL n=3, female sham n=4 and female + MePRDL n=2

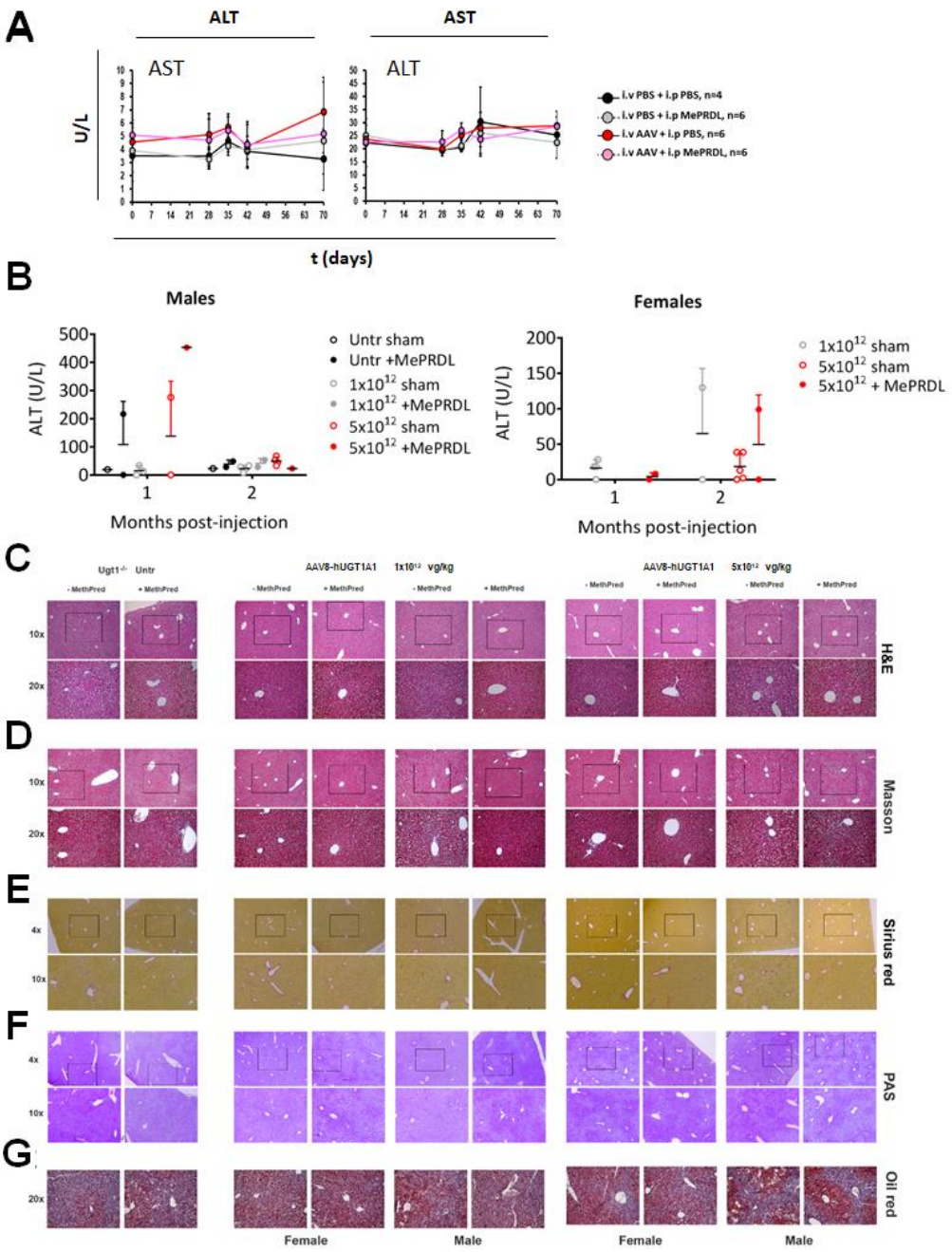

Figure 7.13 Corticosteroid treatment safety in CN animal models.
(A) Plasma transaminases levels in CN rat model. Gunn rats were injected with PBS (black) or 5x10^{12}vg/kg of (ss)AAV8-UGT1A1 (red) at day 0. One month after the treatment, a 15 days tapered dose of methyl-prednisolone (MePRDL) was administered intraperitoneally in half of the rats (grey and pink). Rats were weekly bled and sacrificed 3 months after the first injection. Values of activity of AST and ALT enzymes are plotted versus time. Data are expressed as mean ± standard error. (B-G) Ugt1$^{-/-}$ mice were injected as adults with the indicated doses (vg/kg). Corticosteroid treatment was initiated the day before the injection of the AAV vector and lasted for 5 days at decreasing doses (5.0, 2.5, 1.3, 0.6 and 0.3 mg/kg). Mice were monthly bled and sacrificed 9 months after the first injection. (B) Plasma transaminases levels in CN mouse model. ALT levels (U/m) at 1 and 2 months post-injection, as mean+SD. Each dot represents a single animal. (C) Representative images of H&E; (D) Masson trichrome; (E) Sirius red; (F) PAS and (G) Oil red stainings. Ugt1$^{-/-}$ untreated animals where used as control. Rectangles show magnification

Similar experiments were performed in adult Ugt1$^{-/-}$ mice, treated daily with methylprednisolone starting one day before vector administration **Figure 7.12C**). No adverse effects were noted following methylprednisolone treatment, as correction of TB levels with (ss)AAV8-hUGT1A1 was achieved in mice with or without corticosteroids

(**Figure 7.12D**). Accordingly, liver enzymes in methylprednisolone-treated and control mice remained unchanged (**Figure 7.13B**). Histological analysis of the liver 9 months post injection showed no major abnormalities in mice treated with corticosteroids compared to their controls (**Figure 7.13C-G**). Finally, molecular analysis of VGCN showed no significant effect of corticosteroid on liver transduction (**Figure 7.12E**, p=0.1608, sham vs. MePRDL).

These results demonstrate the safety of glucocorticoids administration concomitant to gene transfer in animal models of CN syndrome.

Concomitant rapamycin treatment and (ss)AAV8 hUGT1A1 gene transfer is safe in rat model of Crigler-Najjar syndrome

We continued our efforts turned to block immune responses and achieve long-term efficacy after AAV-mediated gene transfer to liver, with assessment of the safety of a transient immunosuppression (IS) regimen containing Sirolimus (also named rapamycin, or Rapamune®). This specific IS molecule is of great interest because it has been shown to promote the induction of regulatory T cells,[42-44] essential to establish and maintain transgene tolerance following liver gene transfer with AAV vectors.[45-47]

We sought to determine whether co-administration of rapamycin altered transduction efficiency of (ss)AAV8-hUGT1A1 in Gunn rats. Animals were treated with rapamycin 2 days prior to vector infusion, and continued for 16 days post AAV treatment. Daily observations did not reveal any abnormalities. Correction of TB levels was observed in all animals, with no significant differences between the rapamycin treated group and PBS control (**Figure 7.14A**). Liver enzymes measured in rapamycin-treated and control rats remained unchanged (**Figure 7.14B**). Interestingly, administration of rapamycin at the time of vector infusion resulted in a significant reduction of anti-AAV8 antibody levels and of Nab titers (**Figure 7.14C-D**). This indicates that the immunomodulatory effect of rapamycin is sufficient to reduce significantly capsid immunogenicity. Anti-human UGT1A1 antibody responses were also analyzed. No significant differences were noted in the levels of anti-hUGT1A1 antibodies across treatment groups (**Figure 7.14E**). Additionnaly, at sacrifice of animals, no detrimental effects of rapamycin treatment on VGCN in the liver was noted (**Figure 7.14F**).

These results indicate that administration of rapamycin around the time of gene transfer with (ss)AAV8-hUGT1A1 is safe and does not alter liver transduction efficiency. Additionally, the approach appears to be highly effective in modulating anti-capsid humoral immunity.

3.4 Pharmacokinetics and product metabolism in animals

To support the safety of liver gene transfer with (ss)AAV8-UGT1A1 administered as a single i.v. dose, biodistribution, persistence and shedding were investigated as part of the combined GLP compliant biodistribution/toxicity study in rats and as part of a GLP compliant germline transmission study in rabbits.

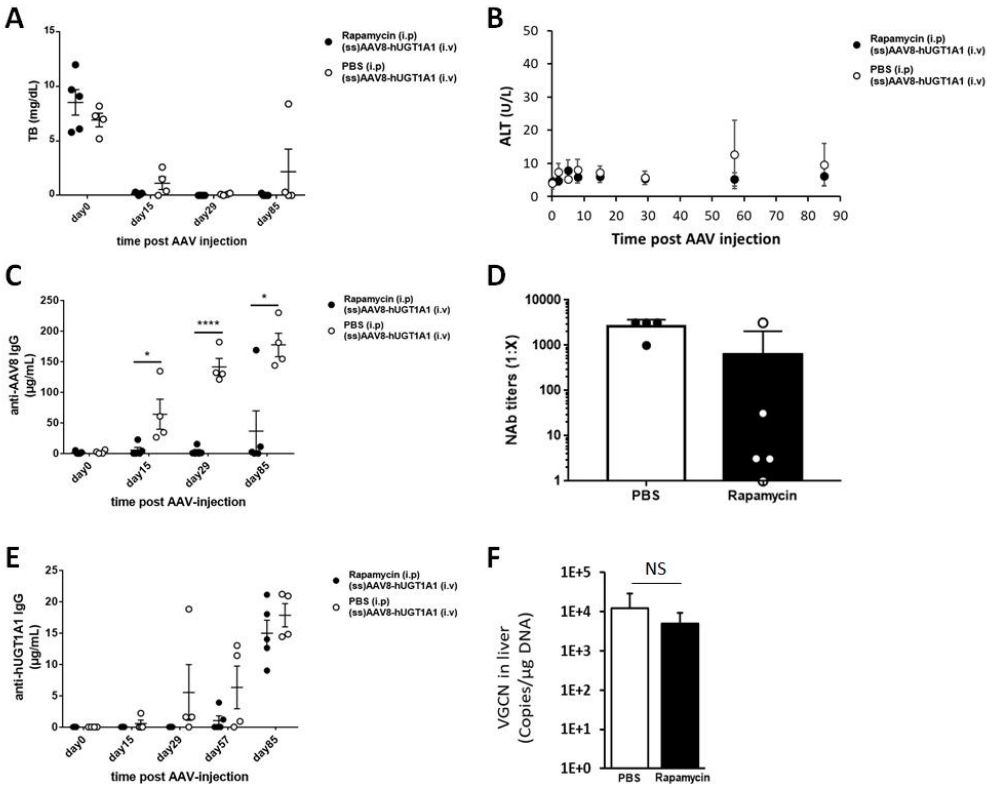

Figure 7.14 Rapamycin treatment in Gunn rats does not affect gene transfer efficacy.
*8 weeks-old Gunn rats were injected in i.p with PBS (n=4) or Rapamycin (1mg/kg; n=5) from two days before (ss)AAV8-hUGT1A1 administration and until 2 weeks post AAV treatment. (ss)AAV8-hUGT1A1 vector was injected in the tail vein at 5x10^{12}vg/kg. The levels of (A) plasma total bilirubin (TB), (B) plasma transaminase (ALT), (C) humoral immune response to AAV8 capsids, (D) anti-AAV8 neutralizing antibodies (Nab), (E) humoral resposne against human UGT1A1 transgene, and (F) Vector Copy number (VGCN) in the liver were analyzed. Rats were sacrificed at day 85. Data are represented in mean ± sd. Statistical analysis were performed by T.test (*p<0.05).*

Biodistribution and long-term persistence of (ss)AAV8-hUGT1A1 in wild-type rats and rabbits

Fisrt, a GLP biodistribution study was performed in wild-type adult Sprague Dawley rats. This was based on the observation that Wistar and Sprague Dawley have similar levels of liver transduction with AAV vectors (data not shown). The aim of this study was to evaluate biodistribution and persistence of the vector genomes after single intravenous administration of (ss)AAV8-hUGT1A1 in wild-type rats with a 26 weeks follow-up. Two vector doses were injected, one equivalent to the maximum planned clinical dose (5x10^{12} vg/kg) and one five times higher (2.5x10^{13} vg/kg).

VGCN and hUGT1A1 mRNA expression levels were measured at day 8, day 91 and day 182 following i.v. administration. No differences in biodistribution were observed between male and female rats. The highest copies of vector were detected in the liver, spleen and lymph nodes, reflecting the tropism of AAV8 vector following i.v. delivery. hUGT1A1 mRNA was detectable on day 8 in all tissues, at levels at least 2-log lower than those measured in the liver, which was the tissue with the highest copies and the highest mRNA expression, reflecting the specificity of the promoter expressing the hUGT1A1 transgene (**Table 7-1** and **Figure 7.15A**). Expression of hUGT1A1 in the liver at day 91 and 182 did not show any significant decrease in transgene expression **Figure 7.15B**).

Table 7-1 Biodistribution and mRNA expression of hUGT1A1 in rats from GLP-biodistributiony study

tissue	day 8 post-AAV injection			
	Vector genome copies /µG DNA		hUGT1A1 RNA copies /µG RNA	
	average	sd	average	se
BR	1.3E+04	5.24E+03	1.9E+03	3.74E+02
CO	1.9E+05	3.38E+05	3.3E+03	5.73E+02
EP	6.2E+04	2.03E+04	1.5E+04	4.26E+03
HE	1.4E+05	4.87E+04	1.6E+04	1.68E+03
KI	1.8E+05	1.06E+05	4.9E+03	2.82E+03
LI	2.3E+06	8.76E+05	2.2E+06	5.86E+05
LLN	8.2E+05	1.98E+05	2.5E+04	1.07E+04
LU	1.0E+05	3.58E+04	5.4E+04	1.03E+04
MLN	1.4E+06	8.58E+05	1.7E+04	4.46E+03
SK	2.1E+05	9.63E+04	1.4E+04	2.71E+03
SM	8.4E+04	3.52E+04	9.7E+03	2.86E+03
SP	7.3E+05	3.48E+05	9.8E+04	3.29E+04
TA	1.2E+06	5.04E+05	5.5E+04	1.92E+04
TE	3.6E+04	2.33E+04	3.8E+03	1.21E+03
UT	2.4E+05	1.10E+05	4.8E+03	3.39E+03

(ss)AAV8-UGT1A1 biodistribution and expression were analyzed by qPCR and RT-qPCR respectively. Results are reported in the table. The number of rats (n) analyzed per each time point was n= 10 (5 males+5 females). Abbreviations : BR brain; CO colon; EP Epididymis; HE Heart; KI Kidney; LI Liver; LLN Lumbar lymph node; LU Lung ; MLN Mesenteric lymph node; SK Skin; SM Skeletal Muscle; SP Spleen; TA Tail (administration site); TE Testis; UT Uterus

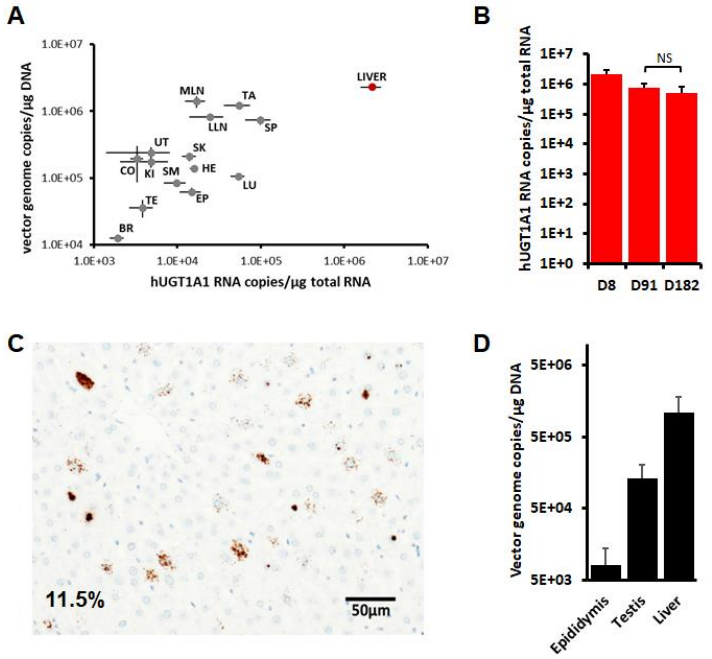

Figure 7.15 (ss)AAV8-hUGT1A1 vector biodistribution.
(A-C) Male and female Sprague-Dawley rats were injected with 5x10^{12} vg/kg or 2.5x10^{13} vg/kg of (ss)AAV8-hUGT1A1 vector produced in suspension at large scale (200L). (A) Correlation of vector genome copy number and hUGT1A1 mRNA expression in different tissues obtained from animals injected with (ss)AAV8-hUGT1A1 vector at 2.5x10^{13} vg/kg. (B) hUGT1A1 mRNA expression measured in liver 8, 91 and 182 days after vector injection. Mean ± SD is shown. Statistical analyses were performed by ANOVA (NS, not significant). (C) In-situ hybridization analysis of hUGT1A1 mRNA in liver of rats injected with (ss)AAV8-hUGT1A1 vector at 2.5x10^{13} vg/kg. (D) Male rabbits were injected with 5x10^{12} vg/kg of (ss)AAV8-hUGT1A1 vector or PBS. Vector genomes were quantified by qPCR in epididymis, testis and liver samples collected at Day 150. Mean ± SD are shown.

Transgene expression was further confirmed by *in situ* hybridization (ISH) in rat liver tissue at day 182 (**Figure 7.15C**), showing ~11.5% of hepatocytes expressing the UGT1A1 transgene.

A separate GLP study was aimed at assessing the risk of inadvertent germline transmission in male animals. (ss)AAV8-hUGT1A1 was administered to male rabbits at a dose of $5x10^{12}$ vg/kg. Vector genomes were determined by qPCR in liver, testis and epididymis, collected at day of sacrifice, 150 days after treatment (**Figure 7.15D**). VGCN were quantifiable in all tissues samples from treated animals collected at Day 150, with the most positive tissue being the liver.

All together, these results indicate the long-term persistence of the (ss)AAV8-hUGT1A1 genome in all analyzed organs in two different animal models, with the highest levels detected in the liver, as reflect of a normal AAV8 tropism following i.v. delivery. The highest expression of the hUGT1A1 transgene was detected in the target organ, the liver, and persisted for a period of at least 150 days in rabbits and 182 days in rats.

Transient shedding of (ss)AAV8-hUGT1A1 in wild-type rats and rabbits

As part of the GLP compliant studies, vector shedding through blood and urine in rats and through sperm in rabbits were assessed. In rats, following i.v. administration, the vector was cleared from blood and urine within 29 days with levels of vector genomes completely undistinguishable from the limit of quantification (LOQ) of the measurement method (**Figure 7.16A-B**). Vector shedding in rabbit sperm was evaluated pre-treatment and from day 3 to day 150 post-treatment. Genomes were quantifiable from Day 3 to Day 30 and became completely undetectable thereafter (**Figure 7.16C**).

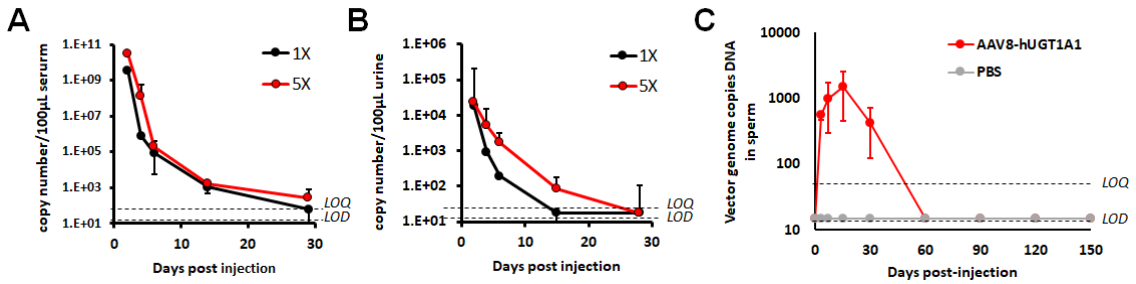

Figure 7.16 (ss)AAV8-hUGT1A1 vector shedding.
(A-B) Male and female Sprague-Dawley rats were injected with $5x10^{12}$ vg/kg (1X) or $2.5x10^{13}$ vg/kg (5X) of (ss)AAV8-hUGT1A1 vector produced in suspension at large scale (200L). Vector shedding in fluids collected from D2 to D29. Vector genome copy number detected in serum (A) and urine (B). Limit of detection (LOD) and limit of quantification (LOQ) of the assay are indicated. (C) Male rabbits were injected with $5x10^{12}$ vg/kg of (ss)AAV8-hUGT1A1 vector or PBS. Vector shedding in sperm collected from D3 to D150. Mean ± SD of vector genome copy number detected in sperm are shown. Limit of detection (LOD) and limit of quantification (LOQ) of the assay are indicated.

These results indicate that the vector shedding was only transient, showing clearance of (ss)AAV8-hUGT1A1 vector genome from fluids within a month, after a single intravenous injection at doses up to $2.5x10^{13}$ vg/kg. Results obtained in male rabbits also indicate that the risk for germline transmission at the doses tested is likely to be very low (see also further).

3.5 Toxicology

Absence of toxicity in wild-type rats treated with (ss)AAV8-hUGT1A1

Toxicity of (ss)AAV8-hUGT1A1 vectors following a single intravenous administration was assessed in the frame of the previous mentioned combined GLP biodistribution/toxicity study in male and female Sprague-Dawley rats.

No treatment-related effects on clinical signs, body weights and body weight changes, food consumption and water consumption, ophthalmology, haematology, coagulation, clinical chemistry (**Table 7-2**), urinalysis and macroscopic findings were noted at Days 8, 91 or 182 post-administration.

Table 7-2 GLP toxicology study : Biochemistry and Hematology data

BIOCHEMISTRY												
	MALES						FEMALES					
		day 8			day 91			day 8			day 91	
ASSAY	VEHICLE	1X	5X	VEHICLE	1X	5X	VEHICLE	1X	5X	VEHICLE	1X	5X
AST (U/L)	90±7	73±12	74±5	85±11	75±8	84±26	78±6	63±5	64±5	70±13	75±9	75±15
ALT (U/L)	78±8	68±14	69±12	59±7	55±7	66±26	53±15	49±9	43±10	53±18	53±13	50±10
ALP (U/L)	280±39	265±43	290±21	103±13	101±15	104±14	190±27	136±32	176±27	61±10	70±16	57±7
GGT (U/L)	2±0	2±0	2±0	2±0	2±0	2±0	2±0	2±0	2±0	2±0	2±0	2±0
CK (U/L)	229±26	238±22	224±36	199±117	136±20	176±63	159±14	193±65	225±20	109±18	129±43	165±75
TBIL (µmol/L)	1.3±0.0	1.3±0.0	1.3±0.0	1.3±0.0	1.3±0.0	1.3±0.0	1.3±0.0	1.3±0.0	1.3±0.0	1.3±0.0	1.3±0.0	1.3±0.0
UREA (mmol/L)	5.1±0.6	4.3±0.1	4.0±0.3	5.4±0.8	5.2±0.6	4.8±0.4	4.4±0.3	4.6±0.3	3.8±0.5	5.1±0.8	5.0±0.8	5.2±0.3
CREAT (µmol/L)	23±1	21±2	16±6	31±4	30±2	29±3	24±2	23±3	26±5	35±4	36±2	35±3
GLUC (mmol/L)	8.58±1.17	18.81±2.91	11.82±2.33	9.65±1.56	9.45±1.91	9.80±2.14	8.67±0.48	10.32±1.14	10.71±1.17	9.18±0.78	9.34±0.85	9.54±1.52
CHOL (mmol/L)	2.2±0.4	2.1±0.9	2.1±0.3	1.5±0.2	1.6±0.3	1.7±0.4	1.9±0.3	2.0±0.6	1.8±0.3	1.8±0.4	1.9±0.4	1.7±0.4
TRIG (mmol/L)	1.19±0.35	1.01±0.38	1.43±0.42	1.18±0.51	1.25±0.38	1.44±0.49	0.44±0.13	0.86±0.44	0.61±0.23	0.83±0.27	0.94±0.43	0.77±0.21
TPROT (g/L)	54±2	54±2	54±1	59±2	60±2	62±1	57±2	57±2	56±3	65±3	68±4	67±4
ALB (g/L)	41±2	40±3	40±1	41±2	42±1	41±2	45±2	43±2	42±3	50±3	52±3	51±3
GLOB (g/L)	13±1	14±3	13±1	18±2	19±1	21±2	13±1	14±2	13±1	15±2	16±2	16±2
A/G	3.1±0.5	2.9±0.8	3.0±0.2	2.3±0.3	2.2±20.1	2.0±0.2	3.6±0.3	3.3±0.5	3.2±0.3	3.5±0.6	3.4±0.6	3.2±0.4
CA (mmol/L)	2.77±0.08	2.76±0.04	2.74±0.07	2.52±0.11	2.55±0.08	2.60±0.06	2.72±0.07	2.69±0.09	2.68±0.07	2.61±0.08	2.64±0.11	2.62±0.13
PHOS (mmol/L)	2.14±0.25	2.77±0.22	2.54±0.23	1.17±0.21	1.28±0.17	1.11±0.13	2.02±0.11	2.41±0.17	2.69±0.13	1.09±0.26	1.10±0.35	1.01±0.18
NA (mmol/L)	144±1	142±2	143±1	143±1	143±1	143±1	143±2	142±1	141±0	142±1	143±1	143±2
K (mmol/L)	4.5±0.2	4.9±0.4	4.7±0.4	4.4±0.4	4.3±0.2	4.5±0.4	3.9±0.2	4.0±0.1	4.1±0.2	3.8±0.2	3.8±0.4	3.9±0.2
CL (mmol/L)	101±1	100±2	100±1	103±2	103±1	103±2	103±2	101±1	101±1	103±1	104±2	104±3

HEMATOLOGY												
	MALES						FEMALES					
		day 8			day 91			day 8			day 91	
ASSAY	VEHICLE	1X	5X	VEHICLE	1X	5X	VEHICLE	1X	5X	VEHICLE	1X	5X
WBC x10³/L	18.86±1.13	13.7±3.3	14.51±4.93	9.95±3.1	9.49±2.66	10.03±2.54	11.16±3.92	11.39±3.96	10±1.45	5.79±1.19	6.34±1.68	5.93±1.41
NEUT x10³/L	1.6±0.47	1.52±0.6	1.24±0.48	1.22±0.39	1.21±0.45	1.26±0.33	1.17±0.58	0.58*±0.17	0.6±0.19	0.52±0.16	0.65±0.24	0.61±0.2
LYMPH x10³/L	11.86±1.17	11.66±2.6	12.67±4.46	8.28±2.7	7.87±2.4	8.33±2.45	9.62±3.19	12.41±3.75	8.96±1.19	5.01±1.07	5.41±1.52	5.05±1.28
MONO x10³/L	0.31±0.05	0.27±0.11	0.35±0.21	0.21±0.1	0.17±0.09	0.18±0.07	0.16±0.1	0.14±0.03	0.22±0.08	0.11±0.05	0.11±0.04	0.1±0.04
EOS x10³/L	0.31±0.05	0.1±0.01	0.09±0.04	0.13±0.06	0.13±0.04	0.13±0.04	0.09±0.03	0.11±0.04	0.09±0.03	0.1±0.02	0.09±0.04	0.1±0.02
BASO x10³/L	0.06±0.01	0.06±0.04	0.07±0.03	0.03±0.02	0.03±0.02	0.03±0.02	0.04±0.03	0.06±0.03	0.03±0.01	0.02±0.01	0.02±0.01	0.01±0
LUC x10³/L	0.09±0.04	0.09±0.03	0.1±0.05	0.08±0.05	0.09±0.04	0.09±0.03	0.08±0.05	0.1±0.05	0.1±0.03	0.05±0.02	0.06±0.04	0.05±0.01
RBC x10¹²/L	6.35±0.2	6.66*±0.11	6.62*±0.14	8.15±0.37	8.3±0.44	8.48±0.33	6.67±0.18	6.66±0.46	6.51±0.19	7.8±0.16	7.68±0.32	7.74±0.25
HGB g/dL	12.7±0.3	13.2±0.4	13.2±0.3	14.2±0.6	14.4±0.5	14.4±0.5	13±0.4	13.4±0.7	12.7±0.5	14±0.4	14±0.4	14±0.5
HCT L/L	0.397±0.008	0.412±0.015	0.412±0.005	0.438±0.015	0.448±0.013	0.446±0.014	0.392±0.016	0.407±0.021	0.384±0.017	0.425±0.013	0.421±0.014	0.427±0.01
MCV fL	62.4±1.7	61.9±1.6	61.9±0.9	58.8±1.9	54±1.6	52.6±1.2	58.7±1.9	61.2±2.8	59±2.3	54.5±1.7	54.8±0.9	55.2±1.7
MCH pg	19.9±0.5	19.8±0.5	19.9±0.2	17.5±0.5	17.4±0.4	17±0.6	19.5±0.5	20.2±0.8	19.5±0.6	18±0.5	18.2±0.4	18.1±0.4
MCHC g/dL	31.9±0.1	31.9±0.3	32.2±0.6	32.5±0.7	32.2±0.5	32.3±0.5	33.2±0.4	33±0.3	33±0.5	33±0.4	33.2±0.4	32.9±0.5
RDW %	12.5±0.2	13.1±0.5	12.9±0.6	13.6±1.6	13.8±0.9	13.9±0.8	11.8±0.3	11.3±0.2	12.1±0.7	11.5±0.4	11.6±0.3	11.4±0.3
PLT x10³/L	947±123	917±88	878±49	861±186	955±86	830±217	985±28	982±105	972±63	782±84	754±142	803±78
RETIC x10³/L	410.7±38	434±73.6	452.3±91.1	262.4±114.8	256.6±74.9	253.6±58.1	292.6±67	285.4±54.8	329.9±90	172.1±35.7	178.1±34	170.3±37.1

*See Material and Methods for details on the design of the GLP toxicology/biodistribution study. For all parameters, n=5 for day 8 and n=10 for day 91; vehicle group at day 91 n=9. Data sets are reported as mean ± standard deviation. 1X corresponds to animals injected with $5x10^{12}$ vg/kg of (ss)AAV8-hUGTA1; 5X corresponds to animals injected with $2.5x10^{13}$ vg/kg of (ss)AAV8-hUGTA1 * Significantly different from control group (vehicle), p value ≤0.05 (Dunnet)*

No toxicity deriving from low-level ectopic expression of hUGT1A1 was evidenced. No treatment-related microscopic findings were identified on Days 8 and 91 post-treatment. Miscellaneous statistically significant differences between the control and treatment groups were noted; however, these were minor, considered as incidental, had no dose-dependent relationship, and were within normal biological variation for the Sprague Dawley rat strain.

Overall, (ss)AAV8-hUGT1A1 vector was well tolerated at doses up to $2.5x10^{13}$ vg/kg, with no evident signs of toxicity.

Reproductive toxicity is considered extremely low after (ss)AAV8-hUGT1A1 treatment

Persistence of vector at low levels (VGCN <0.1 per diploid genome) in the ovaries and testes of treated rats was detected 6 months post-treatment. Based on this, although the risk of germline transmission is expected to be low, we used ISH to further investigate the exact localization of the vector within the gonads. While a signal was localized in ovaries of rats injected with the highest vector dose, positivity was detectable only within the stroma and in blood capillaries. No staining was observed in any oocytes indicating that germinal cells were negative **(Figure 7.17**). These results indicated that the risk of oocyte transduction by AAV8 vectors and consequent generation of transgenic progenies is considered as extremely low and not relevant for the clinical use of (ss)AAV8-hUGT1A1.

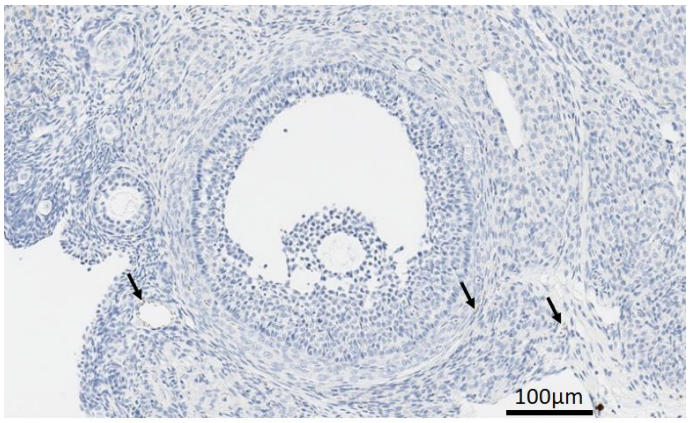

Figure 7.17 (ss)AAV8-hUGT1A1 does not transduce rats oocytes.
In-situ hybridization analysis of hUGT1A1 mRNA in ovary of rats injected with (ss)AAV8-hUGT1A1 vector at $2.5x10^{13}$ vg/kg. Black arrows show exemple of positive cells, in interstitial cells/capillary blood vessel walls/degenerated corpora lutea, suggestive of macrophages and/or endothelial cells and/or smooth muscle cells.

In summary, due to these results and to those demonstrating lack of transduction of sperm cells and complete clearance from sperm following (ss)AAV8-hUGT1A1 vector delivery, inadvertent germline transmission is considered extremely low.

In mice and rats, (ss)AAV8-hUGT1A1 drives the development of humoral responses directed to the human transgene

Humoral immune responses against AAV8 and hUGT1A1 were measured in the context of the GLP toxicology study in rats. All animals developed a significant humoral immune response against the AAV8 capsid starting at day 8 and regardless of the dose infused (**Table 7-3**). Some animals also developed binding IgG against the human UGT1A1 protein (**Table 7-3**).

Table 7-3 GLP toxicology study, humoral immune response against AAV8 capsid and hUGT1A1 transgene

Vector Dose	Anti-AAV8 Capsid binding IgG (mean ± SD; µg/mL)				
	Baseline	day 8	day 29	day 91	day 182
Low dose	0 ± 0.1	38.9 ± 11.3	539.3 ± 161	709.1 ± 16.8	-
High dose	0.1 ± 0.2	26.9 ± 11.8	631.4 ± 93.4	670.7 ± 39.7	1035.9 ± 470.5

Vector Dose	Anti-UGT1A1 transgene binding IgG (mean ± SD; µg/mL)				
	Baseline	day 8	day 29	day 91	day 182
Low dose	0.3 ± 0.5	0.5 ± 0.7	2.4 ± 1.3	16.1 ± 11.9	-
High dose	0.1 ± 0.2	0.7 ± 0.5	3.5 ± 4.2	11.7 ± 8.3	11.8 ± 7.7

Detection of humoral response directed against capsid and transgene in rat plasma. See Material and Methods for details on the design of the GLP toxicology and biodistribution study.

To understand the origin of this response, we measured the formation of anti-transgene antibodies in Ugt1$^{-/-}$ deficient mice treated with AAV8 vector carrying the mouse UGT1A1 transgene (mUGT1A1). Total bilirubin in plasma, transgene expression and humoral response directed against the transgene were assessed. Long-term correction of TB was observed in both male and female animals (**Figure 7.18A**), along with vector genome persistence (**Figure 7.19A**) and UGT1A1 protein expression in liver of treated animals (**Figure 7.18B**, **Figure 7.19B**). No anti-mUGT1A1 binding antibodies were detected in plasma of treated mice one month post injection, while 3 out of 4 of the mice dosed with the human construct (hUGT1A1) had detectable antibodies (**Table 7-4**).

Table 7-4 Testing of species-specific UGT1A1 transgene immunogenicity

UGT1A1 transgene	Number of animals positive for anti-UGT1A1 IgG/ total	
	mice	rats
Human	3/4	2/4
Mouse	0/4	Non tested
Rat	Non tested	0/4

Similar results were obtained in Gunn rats. AAV8-UGT1A1 vectors encoding for both human and rat transgene corrected plasma TB (**Figure 7.18C**). VGCN analysis demonstrated that the two vectors transduced hepatocytes (**Figure 7.19C**) and similar expression of the UGT1A1 protein was observed (**Figure 7.19D**). Anti-human UGT1A1 binding antibodies were measured by ELISA (**Figure 7.18D**) and anti-rat UGT1A1 binding antibodies were measured by Western blot (**Figure 7.18E**). Also in this case, the species-specific version of the UGT1A1 transgene appeared to be less immunogenic than the human counterpart (**Table 7-4**).

These results indicate that the human UGT1A1 transgene elicits humoral responses in rodents, supporting the use of species-specific transgenes to more carefully assess transgene immunogenicity.

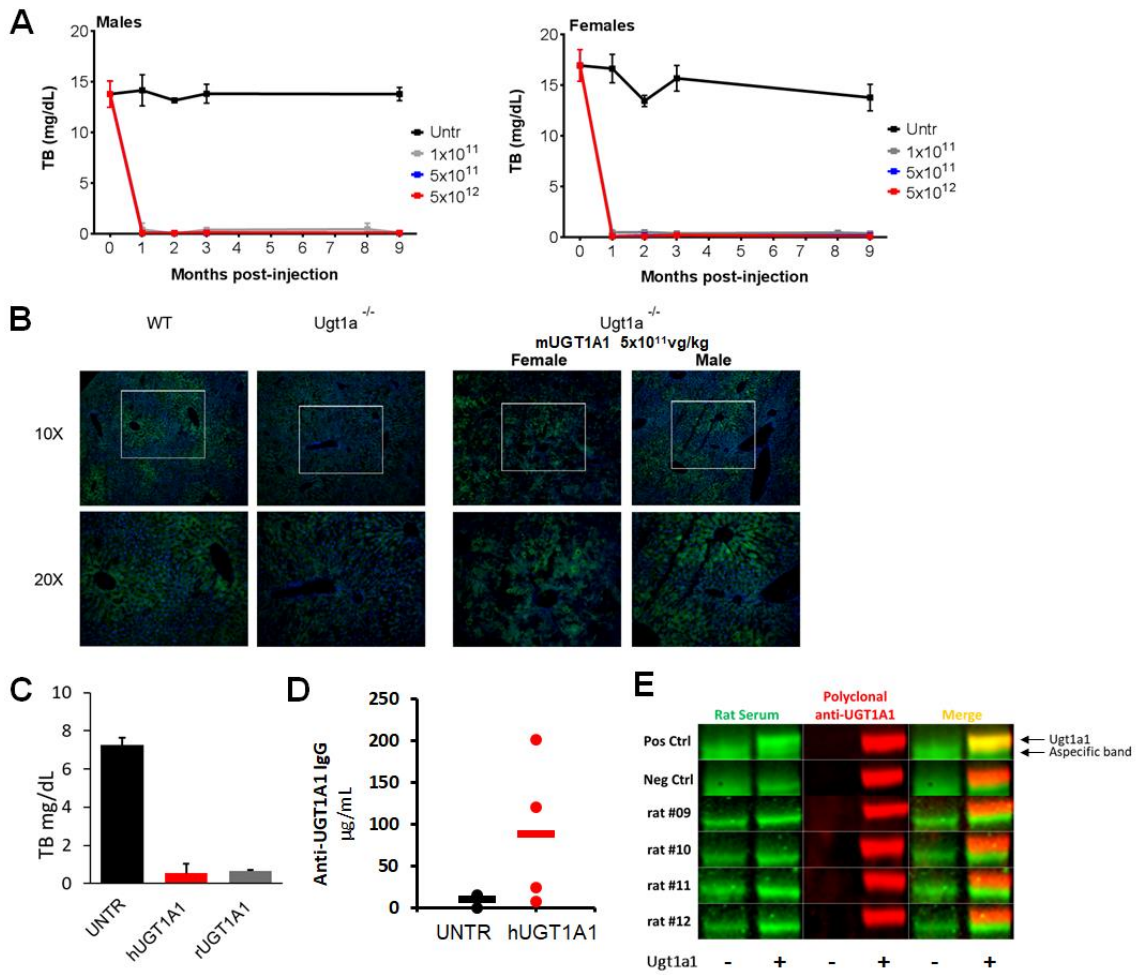

Figure 7.18 Assessment of immunogenicity of species-specific UGT1A1 in two animal models of CN.
(A-B) Ugt1$^{-/-}$ mice (P60) were injected i.p. with PBS (UNTR) or (ss)AAV8-UGT1A1 vectors coding for mouse transgene at 1x10^{1}1 vg/kg, at 5x10^{1}1 vg/kg or 5x10^{12} vg/kg. (A) Plasma bilirubin determination in male and female Ugt1-/- mice after treatment with different doses of AAV8-mUGT1A1 vectors. (B) Immunofluorescence analysis of representative liver sections from WT, Ugt1$^{-/-}$ untreated and treated with 5x10^{11} vg/kg vector dose. Rectangles show magnification images shown below. 1x10^{11}dose male n=5 and female n=3; 5x10^{11} dose male n=3 and female n=4; 5x10^{12} dose male n=3 and female n=4 (C-E) Eight-week-old Gunn rats were injected i.v. with PBS (UNTR) or 5x10^{12} vg/kg of (ss)AAV8-UGT1A1 vector encoding for human UGT1A1 (hUGT1A1) or rat UGT1A1 (rUGT1A1). (C) TB levels in plasma of rats analyzed 186 days post treatment. Data are plotted as mean ± SD. Statistical analyses were performed by ANOVA (NS, not significant). (D,E) Anti-UGT1A1 IgG analyzed 186 days post treatment by ELISA for hUGT1A1 (D) or by Western-Blot for rUGT1A1 (E). Rat livers expressing rUGT1A1 (+) or not (-) were loaded. Individual serum of rat treated with (ss)AAV8-rUGT1A1 (green) or commercial anti-UGT1A1 antibody (red) were used to detect UGT1A1 protein

(ss)AAV8-hUGT1A1 integration sites analysis revealed low risks of genotoxicity and carcinogenicity

For a gene therapy medicinal product, a concern for genotoxicity/carcinogenicity could arise from potential insertional mutagenesis. In this frame, a large scale integration sites (IS) study was conducted to evaluate vector persistence and biosafety in the liver samples from UGT1$^{-/-}$ mice, through the identification and characterization of vector integration events within the host genome.

IS analysis has been performed on mouse liver collected 9 months after (ss)AAV8-hUGT1A1 vector administration. First, AAV vector presence was determined by PCR. Then DNA extracts were analyzed by LAM-PCR using two restriction enzymes and by nrLAM-PCR. Electrophoresis of LAM-PCR products revealed a smear in all samples from treated animals analyzed (data not shown). However, because of the specificity of AAV genome,

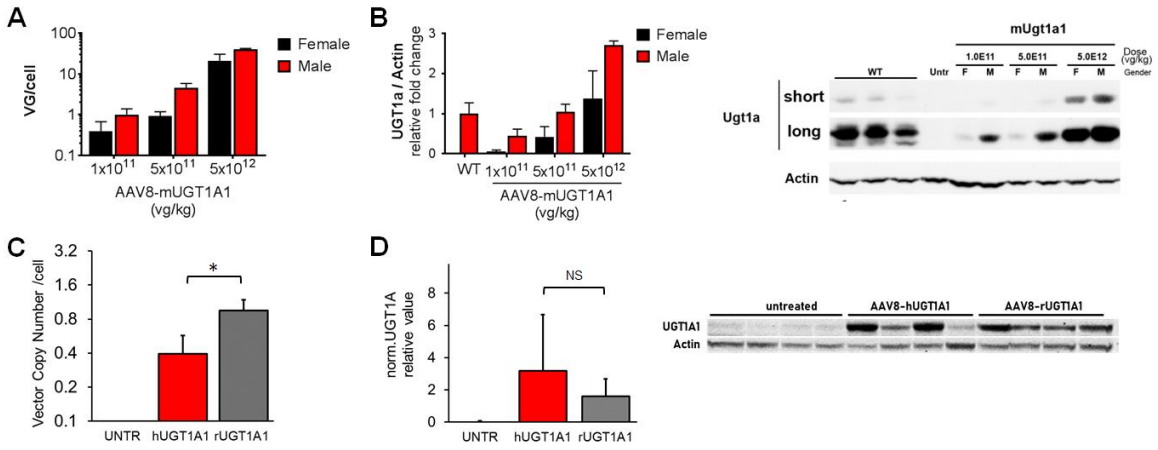

Figure 7.19 Expression of species-specific UGT1A1 transgene in liver of CN rodent models.
(A) Viral genome copy number (VG/cell) in male and female mice 9 months after treatment with different doses of rAAV8-mUGT1A1 and (B) UGT1A1 protein quantification in the liver by Western blot analysis of liver total protein extracts. Endogenous mouse UGT1 protein levels in WT mice where considered as reference. 8 week-old Gunn rats were injected i.v. with PBS (UNTR) or with 5x1012 vg/kg of (ss)AAV8-UGT1A1 vectors encoding for human UGT1A1 (hUGT1A1) or rat UGT1A1 (rUGT1A1). (C) Vector genome copy number, and UGT1A1 protein expression by Western blot (D) in livers of rats analyzed 186 days post treatment. Data are plotted as mean ± SD. Statistical analyses were performed by ANOVA (=p<0.05, NS not significant).*

notably its ability to form circular monomers and rearrangements, the vizualized amplicons derived from both vector-vector and vector-genome (IS) junctions. For this reason, the banding pattern did not allow extrapolating conclusions regarding the clonality of the samples upon AAV administration, prior to high-throughput sequencing.

Then, Illumina MiSeq deep sequencing products (16,495,533 sorted sequence reads) were analyzed in order to identify the different vector blocks present within each individual sequencing read **(Figure 7.20)**. For all samples, sequences bearing one (R1) and two (R2) vector fragments comprised, on average, 92.94 % of the vector-containing reads. These results indicate that the majority of the persisting vector genomes present low levels of rearrangements.

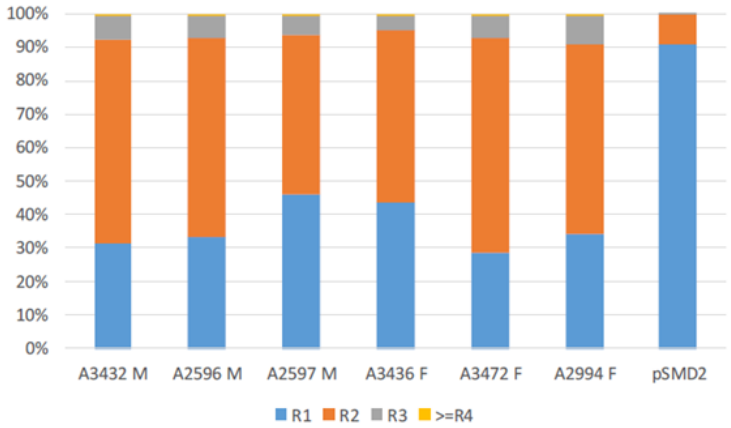

Figure 7.20 Analysis of vector rearrangements on individual liver samples.
Quantification of the detected rearranged vector structures are shown for all transduced samples (n=6) and the plasmid control (pSMD2).

Bioinformatical analyses showed that out of all sorted sequence reads, 424,191 were classified as potential IS. A total number of 1,946 uniquely mappable IS, with low level of

integration frequencies (in the range of 2.10E-4 to 4.30E-4 per cell), were retrieved from mouse liver sample upon (ss)AAV8-hUGT1A1 administration (**Figure 7.21A**) whereas no IS were detected in the pooled samples from control animals (**Figure 7.21B**).

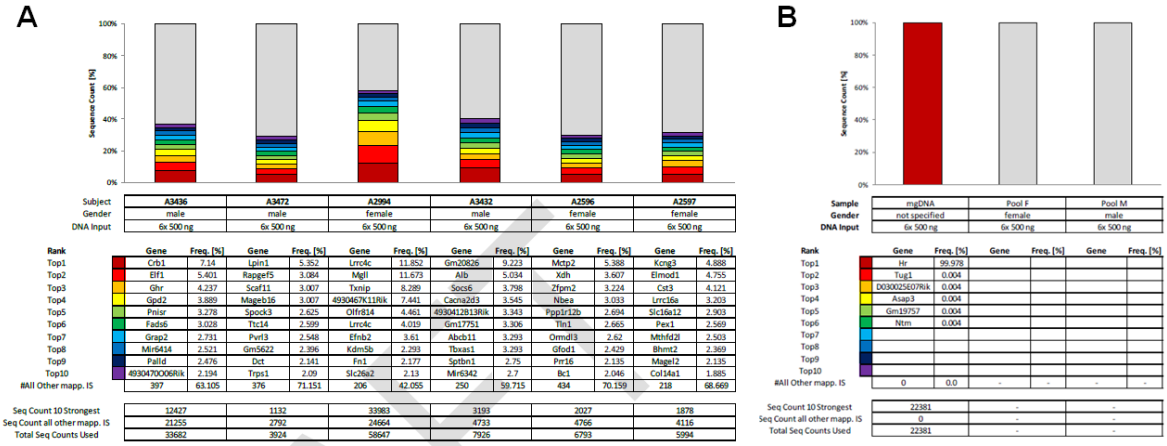

Figure 7.21 Summray of IS annalysis.
Cumulative retrieval frequencies of the ten most prominent IS detected by (nr)LAM-PCR in samples from (A) from vector-treated animals and (B) controls. RefSeq gene names located in vicinity of the IS are given in the table, as well as the relative contribution of each IS to the total read count is presented. The IS detected in the untransduced mouse genomic DNA control (mgDNA) showed no overlap with any of the samples.

In addition, relative distribution of IS among chromosomes (**Figure 7.22A**), within gene coding regions (**Figure 7.22B**) and relative to transcription start sites (**Figure 7.22C**) for all samples, confirm that there was no preferred integration of (ss)AAV8-hUGT1A1 genome and no IS clustering. Importantly, none of the most prominent IS have been detected in more than one sample. Actually, in IS data obtained from all of the analyzed samples, a total of 473 IS (24.3 %) have been found to form 189 common IS (CIS) in the dataset. Regions among top CIS identifed (**Figure 7.22D**), were in line with the literature referring to AAV8, with the presence of the albumin locus in the list.[31, 48]

None of the AAV integration hotspots was located within genes previously associated with AAV-related hepatocellular carcinoma development.

These results demonstrate a low level of IS frequency and no intense CIS formation. These findings do not indicate any vector-mediated genotoxicity in the analyzed samples and point to the safety of the (ss)AAV8-hUGT1A1 gene therapy product.

4. Discussion - conclusion

Gene therapy with AAV vectors holds the potential for a curative treatment for several liver metabolic disorders.[10] Among them, Crigler-Najjar syndrome appears to be an ideal candidate.[8]

Here we propose the use of a single stranded AAV8 vector driving the liver-specific expression of hUGT1A1 for the safe and efficient correction of hyperbilirubinemia, the pathological hallmark of CN syndrome.

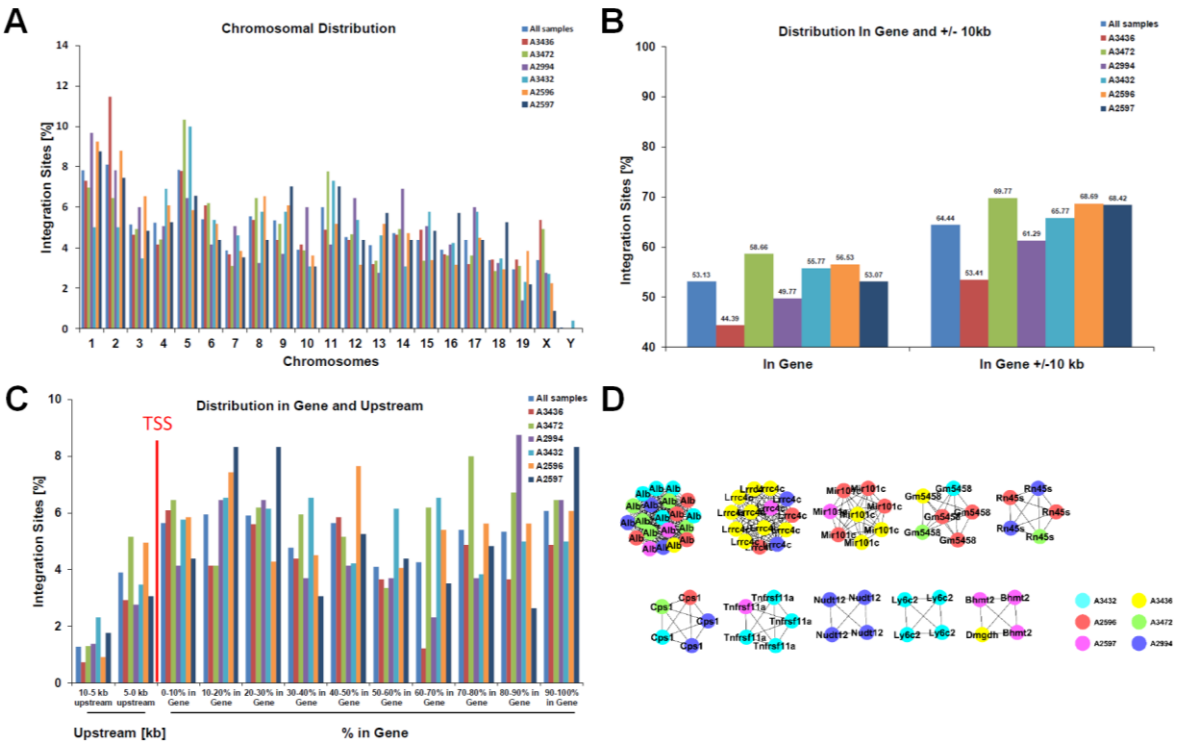

Figure 7.22 IS relative frequency and top10 common IS detected in the dataset
Relative frequency of IS (A) among chromosomes (B) within RefSeq genes and within a 10 kb window around RefSeq genes, (C) upstream of transcription start sites (TSS) and in relative positions within RefSeq genes. (D) Visualization of the Top10 CIS detected in all samples. Each node represents a single unique IS. RefSeq names of closest genes are shown for each IS. Node colors correspond to the respective samples as shown on the right.

In order to select the best candidate for clinical development, we compared a (sc)AAV8 vector designed for the expression of hUGT1A1 with an optimized (ss)AAV8 vector.[21] (sc)AAV and (ss)AAV vectors have been used to transduce the liver in the context of several genetic disorders.[10] To this aim, the use of (sc)AAV vectors could provide potential advantages related to the bypass of the step of dsDNA conversion of the vector genome, thus facilitating the viral transduction.[49] However, the use of (sc)AAV vectors has potential drawbacks, including the reduced genome packaging capacity, the lower vector production yields, and the low homogeneity at least in the context of the current study) due to the production of vectors containing truncated genomes. Hence, here we carefully characterized (sc)AAV8-hUGT1A1 and (ss)AAV8-hUGT1A1 vectors and we showed that, using research-grade production method, (ss)AAV8-hUGT1A1 vectors were generated with higher yields and superior product homogeneity.

Several studies have been published in which both (sc) and (ss) AAV vectors were used to transduce the human liver, these include trials for hemophilia B,[12, 13, 50] hemophilia A,[14] and acute intermittent porphyria.[51] In the present work, we demonstrated in the Gunn rat model of CN, that the clinical candidate (ss)AAV8-hUGT1A1 vector has a similar potency than a (sc)AAV vector carrying the same transgene, both in term of therapeutic efficacy and liver transduction level. One caveat of the current work is that, due to the size of the UGT1A1 transgene and the reduced genome packaging capacity of self-complementary AAVs (~2.5 Kb), the expression cassettes of the (ss) and (sc) AAV8-hUGT1A1 vectors were not identical. Future studies with vectors carrying exactly the same expression cassettes will help to more carefully compare the potency of vectors carrying different genome configurations. Considerations over the ability to produce (sc)AAV vectors in sufficient quantity and of acceptable quality, and concerns over the potential higher immunogenicity of these vectors,[52]

prompted us to focus on a (ss)AAV8-hUGT1A1 vector to develop a scalable production process specific for this vector construct. A scalable animal product-free GMP manufacturing-compliant process for (ss)AAV8-hUGT1A1 vectors production was developed. The process consisted of transient tri-transfection of HEK293 cells cultured in suspension in bioreactors, which was able to supply vector in sufficient quantities to support preclinical and clinical development of the investigational gene therapy candidate.[53] One difference between the suspension manufacturing method and the research-grade production system used in this study resides in the downstream process, which in the suspension process consists of a scalable column-based affinity purification technique, no longer efficient in eliminating the empty particles in the final product. As already highlighted [54], the consequences of the presence of empty capsids in the final product remain unclear, although preparations of AAV vectors containing both capsid species have been safely used in several liver gene transfer clinical trials.[11-15] Additionally, it has been shown that the optimization of the ratio of full to empty capsids in the final formulation of the therapeutic vector could maximize the efficacy of gene transfer, as the empty capsid can act as decoys for anti-AAV neutralizing antibodies.[55] Here, we compared the potency of (ss)AAV8-hUGT1A1 vectors generated with two production methods, one leading to full AAV particles only and the other leading to a mix of empty and full capsids. We demonstrated a similar potency of the two vector preparations both *in vitro* and *in vivo*. We showed that the (ss)AAV8-UGT1A1 vectors produced in suspension and containing empty particles efficiently correct hyperbilirubinemia in CN mice and reached similar liver transduction levels when compared to research-grade vector preparations composed by full particles only.

One important aspect of the current study is that it validates the rat as an ideal model of liver gene transfer. Through a careful comparison of the AAV vector liver transduction efficiency in rats and mice, we provided evidence that the efficiency of liver transduction of AAV8 vectors in rats is similar to that expected in humans. Conversely, AAV8 vectors transduce the mouse liver with at least a log higher efficiency, thus potentially overstating the therapeutic efficacy of a gene therapy drug candidate. Accordingly, using a (ss)AAV8-hFIX vector, we observed lower liver transduction in Gunn rats than in mice. At the same vector dose ($5x10^{12}$ vg/kg) hFIX transgene expression levels detected in blood of mice were ~100% of those measured in humans. Conversely, the injection of the same vector dose in rats resulted in levels of hFIX in the same range of those described in humans receiving a comparable vector dose ($2x10^{12}$ vg/kg) of a similar AAV8 vector encoding for hFIX.[11, 12] These data support the concept that, when available, rats are animal models suitable for dose-finding studies, in particular for AAV8 vectors, and their use in the current study provided a valuable tool to support the choice of a starting dose of vector to be used in the clinic, which holds the prospective of driving therapeutic efficacy in humans.

Efforts were then focused on the manufacturing process scale-up. (ss)AAV8-hUGT1A1 vectors were produced at a 200-liter scale. Vector preparations were carefully analyzed for both the content of full and empty capsids and DNA contaminants, among others. Notably, for the determination of empty to full capsid ratio, here we used AUC, a method that has been demonstrated to provide a reliable readout.[56, 57]

In the perspective of a gene therapy trial, the potential pharmacodynamic interactions of (ss)AAV8-hUGT1A1 with different molecules were investigated. Notably, it seemed important for us to evaluate that no additional drugs that will be taken by the patients during the trial can interfere with the liver gene transfer efficacy. For that, we tested the safety of the use of phenobarbital (an enzymatic enhancer usually taken by CN II patients), glucocorticoids and rapamycin (classical immunomodulation treatments used in clinic) [11-15, 58], in the context of liver gene transfer for CN syndrome. In fact, it was already reported that

immunosuppression can affect the safety and efficacy of gene transfer.[45, 59] Here we demonstrated *in vitro* that phenobarbital does not affect the efficacy of gene transfer and we evidenced in two animal model of CN syndrome that methylprednisolone and rapamycin administration had no impact on the safety and efficacy of the gene therapy. Additionally, this IS regimen appears to be highly effective in modulating anti-capsid humoral immunity.

GLP-compliant biodistribution studies confirmed the expected tissue distribution of AAV8 vectors. However, due to the low-level persistence of vector genomes in gonads 6 months post vector infusion, a germline transmission study was performed in male rabbits. As already reported in animal models and humans, vector was transiently detected in the sperm of rabbits indicating a negligible risk of germline transmission that could be further mitigated by physical barrier contraception during the clinical trial.[11, 12, 60] Similarly, ISH studies in female rats confirmed that AAV vectors do not transduce efficiently oocytes, as previously shown using a reporter vector.[61]

In our GLP toxicology and biodistribution study, all animals developed a significant humoral immune response against the AAV8 capsid, a result that is largely expected.[62] Some animals also developed IgG against the hUGT1A1 protein. Development of anti-human UGT1A1 antibodies has been also documented following the administration of AAV-hUGT1A1, in studies conducted in CN rats, with no consequence on the vector efficacy.[18, 21] The development of anti-hUGT1A1 antibodies is not considered as toxicologically relevant as no elevations of transaminases or treatment-related histopathological findings were noted in the GLP toxicity study. Nevertheless, we further investigated the origin of this finding by testing whether the immune response was driven by the human transgene expressed in rats. To this aim, we injected rat and mouse UGT1A1 transgenes in the corresponding rat and mouse models of CN syndrome. The absence of humoral response directed against rat UGT1A1 in Gunn rats and mouse UGTA1 in the $Ugt1^{-/-}$ mouse model suggests that the anti-hUGT1A1 antibodies observed in the GLP study in rats originated from species-specific transgene immunogenicity. One important implication of this work is that it indicates that immune responses observed in animal models may be driven by species-specific reactions to the transgene expressed, which in some cases may confound the readout of preclinical studies.

Finally, risk linked to potential insertional mutagenesis was investigated. Although AAV vectors do not integrate efficiently in the liver, integrations of AAV vector genomes in the host chromosomes have already been documented. Li and colleagues estimated the frequency of these integration events in the mouse liver as ~1/2000 vector genomes.[63] In our study conducted with (ss)AAV8-hUGT1A1 on mouse liver samples, the integration frequency was found in the same range, with no preferred integration in genes previously shown to mediate malignant transformation in preclinical studies.[64, 65] Moreover, long-term non-clinical studies with (ss)AAV8-hUGT1A1 were previously conducted in Gunn rats.[19] Animals were followed for one-year post delivery of $5x10^{12}$ vg/kg and histopathological examination was conducted at the end of the study. No lesions in liver or HCC were documented in this analysis and the study did not point towards increased risk for tumor formation in other organs (brain, heart, lungs, diaphragm, spleen, kidneys, intestine, bone marrow). Of note, the promoter used to drive the expression of the transgene in the (ss)AAV8-hUGT1A1 appeared to be safe in genotoxicity studies, where no HCC were documented in both neonate and adult animals injected with AAV vectors carrying this promoter.[63, 64] Thus, the risk of HCC formation following (ss)AAV8-hUGT1A1 can be considered extremely low. Additionnaly, several long-term studies with large animal models such as dogs and non-human primates indicated a relative safe profile of AAV with low integration.[66, 67] Nevertheless, very recently, clonal insertion of AAV genome fragments was

reported in Hemophilia A dogs injected with AAV vectors expressing clotting factor VIII under the liver-specific TBG promoter.[68] Although the insertion occurred in genes previously associated with growth control and transformation in humans, histological analysis did not showed any tumor foci. So far, no information concerning HCC development, due to rAAV insertion, has been documented in patients and given that the number of AAV-treated subjects remains small, further studies are necessary to clarify the impact of AAV gene therapy on HCC development together with a careful follow-up in injected subjects.

In conclusion, this work describes the development of an investigational gene therapy for CN syndrome based on an AAV8 vector injected intravenously to deliver a corrected copy of the UGT1A1 gene to hepatocytes. The vector construct has been optimized for liver-restricted expression and is based on a gene delivery platform with proven excellent safety and efficacy profile in humans. We showed successful and efficient manufacturing scale-up and described a comprehensive approach to safety and efficacy in preclinical studies. This work constitutes the basis for the initiation of an AAV-mediated gene therapy clinical trial in CN.

5. References

1. Crigler, J.F., Jr. & Najjar, V.A. Congenital familial nonhemolytic jaundice with kernicterus. *Pediatrics* **10**, 169-80 (1952).
2. Arias, I.M., Gartner, L.M., Cohen, M., Ezzer, J.B. & Levi, A.J. Chronic nonhemolytic unconjugated hyperbilirubinemia with glucuronyl transferase deficiency. Clinical, biochemical, pharmacologic and genetic evidence for heterogeneity. *Am J Med* **47**, 395-409 (1969).
3. Tu, Z.H. et al. Liver transplantation in Crigler-Najjar syndrome type I disease. *Hepatobiliary Pancreat Dis Int* **11**, 545-8 (2012).
4. Lysy, P.A. et al. Liver cell transplantation for Crigler-Najjar syndrome type I: update and perspectives. *World J Gastroenterol* **14**, 3464-70 (2008).
5. Hafkamp, A.M., Nelisse-Haak, R., Sinaasappel, M., Oude Elferink, R.P. & Verkade, H.J. Orlistat treatment of unconjugated hyperbilirubinemia in Crigler-Najjar disease: a randomized controlled trial. *Pediatr Res* **62**, 725-30 (2007).
6. van der Veere, C.N. et al. Current therapy for Crigler-Najjar syndrome type 1: report of a world registry. *Hepatology* **24**, 311-5 (1996).
7. Fagiuoli, S., Daina, E., D'Antiga, L., Colledan, M. & Remuzzi, G. Monogenic diseases that can be cured by liver transplantation. *J Hepatol* **59**, 595-612 (2013).
8. Junge, N., Mingozzi, F., Ott, M. & Baumann, U. Adeno-associated virus vector-based gene therapy for monogenetic metabolic diseases of the liver. *J Pediatr Gastroenterol Nutr* **60**, 433-40 (2015).
9. Bortolussi, G. & Muro, A.F. Advances in understanding disease mechanisms and potential treatments for Crigler–Najjar syndrome. *Expert Opinion on Orphan Drugs* **6**, 425-439 (2018).
10. Mingozzi, F. & High, K.A. Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. *Nat Rev Genet* **12**, 341-55 (2011).
11. Nathwani, A.C. et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med* **365**, 2357-65 (2011).
12. Nathwani, A.C. et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* **371**, 1994-2004 (2014).
13. George, L.A. et al. Hemophilia B Gene Therapy with a High-Specific-Activity Factor IX Variant. *N Engl J Med* **377**, 2215-2227 (2017).
14. Rangarajan, S. et al. AAV5-Factor VIII Gene Transfer in Severe Hemophilia A. *N Engl J Med* **377**, 2519-2530 (2017).
15. Miesbach, W. et al. Gene therapy with adeno-associated virus vector 5-human factor IX in adults with hemophilia B. *Blood* **131**, 1022-1031 (2018).
16. Bortolussi, G. et al. Rescue of bilirubin-induced neonatal lethality in a mouse model of Crigler-Najjar syndrome type I by AAV9-mediated gene transfer. *FASEB J* **26**, 1052-63 (2012).
17. Bortolussi, G. et al. Life-long correction of hyperbilirubinemia with a neonatal liver-specific AAV-mediated gene transfer in a lethal mouse model of Crigler-Najjar Syndrome. *Hum Gene Ther* **25**, 844-55 (2014).

18. Seppen, J. et al. Adeno-associated virus vector serotypes mediate sustained correction of bilirubin UDP glucuronosyltransferase deficiency in rats. *Mol Ther* **13**, 1085-92 (2006).
19. Ronzitti, G. et al. A translationally optimized AAV-UGT1A1 vector drives safe and long-lasting correction of Crigler-Najjar syndrome. *Mol Ther Methods Clin Dev* **3**, 16049 (2016).
20. Bockor, L. et al. Repeated AAV-mediated gene transfer by serotype switching enables long-lasting therapeutic levels of hUgt1a1 enzyme in a mouse model of Crigler-Najjar Syndrome Type I. *Gene Ther* **24**, 649-660 (2017).
21. Ronzitti, G. et al. A translationally optimized AAV-UGT1A1 vector drives safe and long-lasting correction of Crigler-Najjar syndrome. *Molecular Therapy — Methods & Clinical Development* **3**, 16049 (2016).
22. Collaborators, G.H. et al. Estimates of global, regional, and national incidence, prevalence, and mortality of HIV, 1980-2015: the Global Burden of Disease Study 2015. *Lancet HIV* **3**, e361-87 (2016).
23. Ayuso, E., Mingozzi, F. & Bosch, F. Production, purification and characterization of adeno-associated vectors. *Curr Gene Ther* **10**, 423-36 (2010).
24. Snyder, R.O. et al. Efficient and stable adeno-associated virus-mediated transduction in the skeletal muscle of adult immunocompetent mice. *Hum Gene Ther* **8**, 1891-900 (1997).
25. Thummel, C., Tjian, R. & Grodzicker, T. Construction of adenovirus expression vectors by site-directed in vivo recombination. *J Mol Appl Genet* **1**, 435-46 (1982).
26. Meliani, A. et al. Enhanced liver gene transfer and evasion of preexisting humoral immunity with exosome-enveloped AAV vectors. *Blood Adv* **1**, 2019-2031 (2017).
27. Montenegro-Miranda, P.S. et al. Adeno-associated viral vector serotype 5 poorly transduces liver in rat models. *PLoS One* **8**, e82597 (2013).
28. Schmidt, M. et al. High-resolution insertion-site analysis by linear amplification–mediated PCR (LAM-PCR). *Nature methods* **4**, 1051-1057 (2007).
29. Paruzynski, A. et al. Genome-wide high-throughput integrome analyses by nrLAM-PCR and next-generation sequencing. *Nature protocols* **5**, 1379 (2010).
30. Gabriel, C. et al. Rapid high-throughput human leukocyte antigen typing by massively parallel pyrosequencing for high-resolution allele identification. *Human immunology* **70**, 960-964 (2009).
31. Gil-Farina, I. et al. Recombinant AAV integration is not associated with hepatic genotoxicity in nonhuman primates and patients. *Molecular Therapy* **24**, 1100-1105 (2016).
32. Afzal, S., Wilkening, S., von Kalle, C., Schmidt, M. & Fronza, R. GENE-IS: time-efficient and accurate analysis of viral integration events in large-scale gene therapy data. *Molecular Therapy-Nucleic Acids* **6**, 133-139 (2017).
33. Fronza, R., Vasciaveo, A., Benso, A. & Schmidt, M. A graph based framework to model virus integration sites. *Computational and structural biotechnology journal* **14**, 69-77 (2016).
34. Shannon, P. et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research* **13**, 2498-2504 (2003).
35. Nathwani, A.C. et al. Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* **107**, 2653-61 (2006).
36. Gunn, C.K. Hereditary Acholuric Jaundice in the Rat. *Can Med Assoc J* **50**, 230-7 (1944).
37. Cornelius, C.E. & Arias, I.M. Animal model of human disease. Crigler-Najjar Syndrome. Animal model: hereditary nonhemolytic unconjugated hyperbilirubinemia in Gunn rats. *Am J Pathol* **69**, 369-72 (1972).
38. Sato, H., Aono, S., Kashiwamata, S. & Koiwai, O. Genetic defect of bilirubin UDP-glucuronosyltransferase in the hyperbilirubinemic Gunn rat. *Biochem Biophys Res Commun* **177**, 1161-4 (1991).
39. Davidoff, A.M., Ng, C.Y., Zhou, J., Spence, Y. & Nathwani, A.C. Sex significantly influences transduction of murine liver by recombinant adeno-associated viral vectors through an androgen-dependent pathway. *Blood* **102**, 480-8 (2003).
40. Catz, C. & Yaffe, S. Barbiturate Enhancement of Bilirubin Conjugation and Excretion in Young and Adult Animals [35]. *Pediatric research* **2**, 361-370 (1968).
41. Yaffe, S.J., Levy, G., Matsuzawa, T. & Baliah, T. Enhancement of glucuronide-conjugating capacity in a hyperbilirubinemic infant due to apparent enzyme induction by phenobarbital. *New England Journal of Medicine* **275**, 1461-1466 (1966).
42. Battaglia, M., Stabilini, A. & Roncarolo, M.-G. Rapamycin selectively expands CD4+ CD25+ FoxP3+ regulatory T cells. *Blood* **105**, 4743-4748 (2005).
43. Battaglia, M. et al. Rapamycin promotes expansion of functional CD4+ CD25+ FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. *The Journal of Immunology* **177**, 8338-8347 (2006).
44. Battaglia, M. et al. Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes* **55**, 40-49 (2006).

45. Mingozzi, F. et al. Modulation of tolerance to the transgene product in a nonhuman primate model of AAV-mediated gene transfer to liver. *Blood* **110**, 2334-41 (2007).
46. Nayak, S. et al. Prevention and reversal of antibody responses against factor IX in gene therapy for hemophilia B. *Frontiers in microbiology* **2**, 244 (2011).
47. Keeler, G.D. et al. Gene therapy-induced antigen-specific Tregs inhibit neuro-inflammation and reverse disease in a mouse model of multiple sclerosis. *Molecular Therapy* **26**, 173-183 (2018).
48. Chandler, R.J., LaFave, M.C., Varshney, G.K., Burgess, S.M. & Venditti, C.P. Genotoxicity in mice following AAV gene delivery: a safety concern for human gene therapy? *Molecular Therapy* **24**, 198-201 (2016).
49. McCarty, D.M., Monahan, P.E. & Samulski, R.J. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther* **8**, 1248-54 (2001).
50. Manno, C.S. et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* **12**, 342-7 (2006).
51. D'Avola, D. et al. Phase I open label liver-directed gene therapy clinical trial for acute intermittent porphyria. *J Hepatol* (2016).
52. Martino, A.T. et al. The genome of self-complementary adeno-associated viral vectors increases Toll-like receptor 9-dependent innate immune responses in the liver. *Blood* **117**, 6459-68 (2011).
53. Chahal, P.S., Schulze, E., Tran, R., Montes, J. & Kamen, A.A. Production of adeno-associated virus (AAV) serotypes by transient transfection of HEK293 cell suspension cultures for gene delivery. *J Virol Methods* **196**, 163-73 (2014).
54. Wright, J.F. AAV empty capsids: for better or for worse? *Mol Ther* **22**, 1-2 (2014).
55. Mingozzi, F. et al. Overcoming preexisting humoral immunity to AAV using capsid decoys. *Sci Transl Med* **5**, 194ra92 (2013).
56. Nass, S.A. et al. Universal Method for the Purification of Recombinant AAV Vectors of Differing Serotypes. *Mol Ther Methods Clin Dev* **9**, 33-46 (2018).
57. Burnham, B. et al. Analytical Ultracentrifugation as an Approach to Characterize Recombinant Adeno-Associated Viral Vectors. *Hum Gene Ther Methods* **26**, 228-42 (2015).
58. Mendell, J.R. et al. Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. *N Engl J Med* **377**, 1713-1722 (2017).
59. Montenegro-Miranda, P.S., ten Bloemendaal, L., Kunne, C., de Waart, D.R. & Bosma, P.J. Mycophenolate mofetil impairs transduction of single-stranded adeno-associated viral vectors. *Hum Gene Ther* **22**, 605-12 (2011).
60. Favaro, P. et al. Host and vector-dependent effects on the risk of germline transmission of AAV vectors. *Mol Ther* **17**, 1022-30 (2009).
61. Paneda, A. et al. Effect of adeno-associated virus serotype and genomic structure on liver transduction and biodistribution in mice of both genders. *Hum Gene Ther* **20**, 908-17 (2009).
62. Davidoff, A.M. et al. Comparison of the ability of adeno-associated viral vectors pseudotyped with serotype 2, 5, and 8 capsid proteins to mediate efficient transduction of the liver in murine and nonhuman primate models. *Mol Ther* **11**, 875-88 (2005).
63. Li, H. et al. Assessing the potential for AAV vector genotoxicity in a murine model. *Blood, The Journal of the American Society of Hematology* **117**, 3311-3319 (2011).
64. Chandler, R.J. et al. Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy. *The Journal of clinical investigation* **125**, 870-880 (2015).
65. Donsante, A. et al. AAV vector integration sites in mouse hepatocellular carcinoma. *Science* **317**, 477 (2007).
66. Niemeyer, G.P. et al. Long-term correction of inhibitor-prone hemophilia B dogs treated with liver-directed AAV2-mediated factor IX gene therapy. *Blood, The Journal of the American Society of Hematology* **113**, 797-806 (2009).
67. Nathwani, A.C. et al. Long-term safety and efficacy following systemic administration of a self-complementary AAV vector encoding human FIX pseudotyped with serotype 5 and 8 capsid proteins. *Mol Ther* **19**, 876-85 (2011).
68. Nguyen, G.N. et al. A long-term study of AAV gene therapy in dogs with hemophilia A identifies clonal expansions of transduced liver cells. *Nat Biotechnol* **39**, 47-55 (2021).

Chapter 8

Additional projects and concluding remarks

1. Additional projects

Determined to bring to the clinic safe and efficient treatments for IEMs such as Crigler-Najjar syndrome (CN) and Pompe disease (PD), we have dedicated these last years to explore multiple avenues that allow improving gene therapy efficacy and modulating immunes responses towards gene therapy product. Although not directly addressed in the present thesis, various reasearch papers resulting from these studies, some of which were already published, have been mentioned in this manuscript.

We showed that liver-directed gene transfer with a secretable acid alpha-glucosidase (GAA) transgene is a highly efficient treatment approach to reverse the PD phenotype at the biochemical and functional levels in Pompe mice, with relatively low vector doses.[1] Recently, we confirmed these results in animal models with an advanced disease, early-onset spinal cord degeneration and respiratory defects.[2, 3] We are currently working on a manuscript describing the effect of this gene therapy approach on the complete correction of the skeletal muscle pathology with a partial effect on the central nervous system (CNS) phenotype, contrarily to treatment with enzyme replacement therapy (ERT).[4] These differences in efficacy were driven by the superior enzyme bioavailability induced by gene transfer.

In an effort to increase GAA plasma half-life and GAA uptake in muscle or CNS, we modified the previously described secretable GAA transgene[1] by the addition of heterologous domains (HD). Seven new bioengineered GAA variants were generated and evaluated in wild type and Pompe mice. After systemic delivery, we demonstrated that the new variants led to higher activity of circulating GAA without modifying the immunogenicity of the protein. Moreover, following administration of selected HD-GAA variants, we observed an increase of GAA uptake in muscle and CNS. In particular, an increase of lysosomal GAA and enzyme activity in triceps, brain and spinal cord were reported. In Pompe mice, increased GAA uptake was associated with a slightly better clearance of glycogen in both brain and spinal cord. A manuscript describing these findings is in preparation.

The selection of an adeno-associated virus (AAV) capsid that efficiently transduce hepatocytes in a species evolutionary close to humans, and/or human hepatocytes has been shown to be crucial for the success of liver gene therapy studies.[5-8] Based on this, we successfully combined a highly hepatotropic AAV vector capsid and an optimized GAA transgene expression cassette, named SPK-3006. *In vitro* and *in vivo* studies were realized to characterize SPK-3006 and to determine the biochemical properties of the transgene-derived protein product following liver expression of secretable GAA. Preclinical studies were also performed in rats and non-human primates (NHP). Basically, when compared to AAV8-GAA, SPK-3006 resulted in a 2.5 fold increase in the amount of circulating GAA upon hepatic gene therapy in NHP. It also successfully resulted in enzyme uptake in key PD target tissues (skeletal muscles and CNS), with no significant changes in clinical pathology parameters and no/minimal development of anti-hGAA antibodies[4] (manuscript in preparation). Notably, in NHPs, effective secretion of GAA enzyme in the circulation and enzyme tissues uptake were observed at vector doses that are about 10-100 fold lower than those currently tested in other clinical trials of muscular and neuromuscular diseases (NCT03199469, NCT03375164)[9, 10] and approved for the treatment of spinal muscular atrophy.[9, 11] This treatment strategy is going to be evaluated in a clinical trial now open in late-onset PD (LOPD) patients (NCT04093349), which will provide a first insight on the safety and efficacy of our approach in humans.

In parallel, our work has also focused on the study of the mechanism of liver-mediated transgene tolerance, to exploit this mechanism to achieve efficient and stable gene transfer into skeletal muscle. Indeed, for neuromuscular disorders such as PD, the delivery of a gene therapy product to muscle without inducing strong immune responses against the transgene is still a challenge.[12, 13] In order to address this issue, in recent publications we showed that concomitant injection of liver-targeted and muscle-targeted vectors resulted in the induction of immune tolerance towards the immunogenic muscle-targeted vector.[14, 15] Importantly, we observed that liver gene transfer had the potential of reversing an ongoing anti-transgene immune response, which can be important in the context of treating patients that have previously developed antibodies to ERT. This strategy was further optimized by the design of tandem promoters, which allowed for simultaneous transgene expression in the liver and in the tissue of interest, and induced transgene tolerance.[16] Together these research works highlighted the potential of hepatic gene expression to prevent anti-transgene immune responses in PD.

Additional research was also performed to address the mechanism by which AAV triggers anti-capsid immune responses. In one of these studies we found that the interaction of the AAV capsid with human PBMCs triggers the secretion of cytokines IL-1β and IL-6 from monocyte-related dendritic cells (moDCs), and that anti-capsid humoral responses could be prevented *in vivo* by blocking the secretion of these two cytokines.[17] We also reported a role of natural killer cells in the initiation of anti-capsid immunity.[17]

Pre-existing humoral immunity to AAV is highly prevalent in humans and can profoundly affect the transduction efficiency.[18] This is particularly true for pre-existing neutralizing antibodies (NAbs), detected in a relatively large number of patients' populations. For example, in Duchenne muscular dystrophy and CN patients populations, NAbs directed against AAV8 were found in around 30-40% of screened patients.[19, 20] Consequently, a large proportion of patients is excluded from enrollment in clinical trials. Similarly, vector re-dosing with the available technology is not feasible because of the development of high-titer antibodies following AAV vector administration.

We proposed different strategies to modulate anti-AAV humoral response. In one of them, we developed an AAV-specific plasmapheresis column, which was shown to efficiently and selectively deplete anti-AAV antibodies, without depleting the total immunoglobulin pool from plasma. Using this technology, we showed a nearly complete removal of anti-AAV antibodies from purified human IgG pools and plasma samples, decreasing titers to levels that did not neutralized AAV vectors in our neutralization test.[21]

In another setting, we evaluated the *in vivo* efficacy of the IdeS, a bacterial endopeptidase known to degrade circulating IgG, to eliminate anti-AAV antibodies in the context of gene therapy. In mice passively immunized with intravenous Ig, we showed that IdeS administration decreased circulation anti-AAV antibodies and enabled efficient liver gene transfer. In NHP, IdeS administration prior to AAV infusion resulted in enhanced AAV liver transduction, even after vector re-administration. We also showed that IdeS was reducing anti-AAV antibody levels from human plasma samples *in vitro*.[22]

Overall, these results provided proof-of-concept of potential solutions and tools that could contribute to overcome some of the limitations of AAV-mediated gene transfer in the clinic, associated to immune reactions to gene therapy vectors.

On the same line of thought, we are also evaluating the efficacy of concomitant administration of rapamycin nanoparticles with AAV vector to induce antigen-specific tolerance towards AAV vectors. This work is currently ongoing in the frame of the European research project CureCN and will be the subject of a near future publication. This work is based on our previous study in which we showed that co-administration of rapamycin nanoparticles prevented the induction of high antibody titers and cytotoxic responses to a first-time administration of AAV.[23] This approach allowed for successful liver transduction and transgene expression upon re-administration of the same vector, contrasting with the effects of AAV re-administration in the absence of rapamycin nanoparticles.[23] Of note, the effect showed to be antigen specific, and administration of a different AAV serotype did not result in transgene expression after the second injection. Recently we demonstrated the relevance of the use of rapamycin compared to free rapamycin. In a manuscript recently accepted, we showed that although rapamycin can prevent the induction of NAbs in naive Gunn rats, it has only a poor effectivity after prior exposure to AAV.

To continue evoking works of the European research project CureCN, we recently developed and validated a cell-based, quantitative potency assay that detects both expression and enzyme activity of an AAV8-hUGT1A1 vector, which is currently under clinical evaluation for the treatment of CN.[24] Actually, potency assessment of clinical-grade vectors is crucial to support future marketing authorization and this work represented an important step in the clinical development of a potential future gene therapy drug.

Other CureCN research studies are ongoing to identify at which age long term correction by AAV-mediated gene delivery can be achieved in mouse and rat models of CN. Indeed, as previously highlighted several times in this manuscript, the progressive loss of transgene expression due to liver growth is an important limitation to liver gene transfer in pediatric populations.[25, 26] In this work, we assessed carefully the stability and safety of gene transfer in juvenile animals, which will support future clinical work aimed administrating with gene therapy in CN patients younger than 10 years. Preliminary results suggest that long-term correction of inherited hyperbilirubinemia can be feasible in one month-old animals, and that earlier treatment results in loss of efficacy. How this translates to human age requires additional investigations to compare liver growth rates in children and juvenile animals. These experiments represent the next tasks of the CureCN project.

Finally, we also developed innovative therapeutic tools for other indications such as Cori disease,[27] hemophilia,[28, 29] ornithine transcarbamylase (OTC) deficiency[30] and also CNS disorders.[31]

2. Concluding remarks and future prospects

Part 1: Next generation approaches for Pompe disease treatment

PD is a fatal lysosomal storage disorder and neuromuscular disease, with systemic, multi-organ manifestations. Today, PD remains an unmet medical need, as immunogenicity of recombinant GAA and long-term outcomes of ERT, the current standard of care, point out to the need for better treatments, both for pediatric and adult patients. This is particularly true when considering its poor efficacy in the rescue of respiratory symptoms and emerging CNS manifestations, increasingly documented.[32-35] As reported in this manuscript, gene therapy holds the potential to revolutionize the way to treat PD. In particular, liver-directed gene transfer with a secretable GAA transgene is a highly efficient treatment approach, with

a low immunogenicity profile, and represents an attractive solution, at least on LOPD patients.

Nevertheless, the complexity and diversity of PD, with an infantile-onset disease presenting with clinical features quite different from those of the late-onset disease, has prompted us to improve and optimize our gene-based approaches. We have worked to find solutions to improve the muscular and neurological symptoms of the disease, exploring substrate reduction therapy (SRT) strategy or trying to ameliorate muscle targeting through improved AAV vectors.

Focusing on muscle-targeting gene therapy that could be applied in both pediatric and adult patients, we demonstrated in this manuscript the feasibility of a miRNA-based and genome editing-based SRT using an AAV-mediated gene transfer approach, associated with efficacy to prevent glycogen build up in Pompe mice treated when neonates. The encouraging preliminary results obtained with this strategy could be further improved using highly optimized myospecific vectors, as those developed in the frame of the Myocure consortium and presented in this thesis, or combined with ERT or gene replacement therapy to improve benefits for the PD patients.

The development of products with improved outcomes, i.e. enhanced glycogen clearance in multiple tissues including muscle and CNS and reduced immune reactions, will likely be the most successful intervention for PD. Considering the induction of immunological tolerance to GAA mediated by the liver, AAV-mediated liver gene therapy with secretable GAA still represents one of the best option for PD patients. We further enhanced its efficacy and ameliorated the GAA delivery in muscle and CNS, by using pharmacological chaperones therapy (PCT). We showed that PCT might increase the safety and tolerability of AAV vectors and ameliorate the biodistribution of GAA, including in the CNS. In particular, this strategy combined with vector optimization may help to reduce the vector dose to administer to patients, and reduce the potential dose related immuno-toxicity.

Application of this gene therapy approaches to PD patients will face challenges, such as the high pre-existing immunity to GAA in some patients, the possibility of an altered immune system in severe-onset disease, and the potential need for vector re-administration. Moreover, pre-existing humoral immunity against AAV, highly prevalent in humans, is a limitation in terms of patient eligibility. In this context, investigational immunomodulatory strategies, such as those evoked earlier in this chapter, will come into play.

The time for gene therapy in PD is now.[36] Of course, there is still a lot to understand about this disease and future exploratory clinical work will be needed to unravel its complexities and, we hope, to highlight the benefits of gene therapy.

Part 2: A novel therapy for Crigler-Najjar syndrome

CN is a life-threatening, liver disease that affects about one in a million individuals at birth. Since few years, we devoted all our energies to elaborating a curative gene therapy for this ultra-rare pathology and to making the treatment available to patients. The works collected in this manuscript show the development of an investigational liver-directed gene therapy based on an optimized AAV8 vector injected intravenously to deliver a corrected copy of the UGT1A1 gene to hepatocytes. We described a comprehensive approach to safety and efficacy evaluation in different relevant animal models and we addressed the requirements of competent regulatory Authorities, to start a clinical trial. Led by the CureCN consortium,

this trial was initiated in 4 centers in Europe and has for objectives to, first, assess the safety and tolerance of the AAV8-UGT1A1 vector, and then, evaluate its therapeutic efficacy in severe CN patients.

As evoked for PD, application of this liver-directed gene therapy approach to CN is facing some major challenges, such as immune responses directed against AAV or the loss of efficacy overtime in a developing liver. The CureCN consortium joins forces to overcome these issues and is currently working on strategies that would allow vector re-dosing or reduce the impact of pre-existing immune responses.

This new curative option that we propose holds the potential to considerably improve the quality of life of CN patients by offering them a valid therapeutic alternative. Our long-term objective is to make the treatment accessible for all people living with CN.

Even if the development of a cure is a long process, this thesis is the reflect of our commitment to bring innovative therapies to patients, to transform the potential of gene therapy into the tomorrow's healthcare solution for rare diseases.

The therapeutic strategies and tools developed in this work for PD and CN are examples of advanced therapies that could be also applied to a range of IEMs.

3. References

1. Puzzo, F. et al. Rescue of Pompe disease in mice by AAV-mediated liver delivery of secretable acid alpha-glucosidase. *Sci Transl Med* **9** (2017).
2. Cagin, U. et al. Rescue of advanced Pompe disease in mice with hepatic expression of secretable acid α-glucosidase. *Molecular Therapy* (2020).
3. Colella, P. et al. Gene therapy with secreted acid alpha-glucosidase rescues Pompe disease in a novel mouse model with early-onset spinal cord and respiratory defects. *EBioMedicine* **61**, 103052 (2020).
4. Armour, S. et al. O. 6Pre-clinical development of SPK-3006, an investigational liver-directed AAV gene therapy for the treatment of Pompe disease. *Neuromuscular Disorders* **29**, S39 (2019).
5. Davidoff, A.M. et al. Comparison of the ability of adeno-associated viral vectors pseudotyped with serotype 2, 5, and 8 capsid proteins to mediate efficient transduction of the liver in murine and nonhuman primate models. *Mol Ther* **11**, 875-88 (2005).
6. George, L.A. et al. Hemophilia B gene therapy with a high-specific-activity factor IX variant. *New England Journal of Medicine* **377**, 2215-2227 (2017).
7. Nathwani, A.C. et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med* **365**, 2357-65 (2011).
8. Nathwani, A.C. et al. Long-term safety and efficacy following systemic administration of a self-complementary AAV vector encoding human FIX pseudotyped with serotype 5 and 8 capsid proteins. *Mol Ther* **19**, 876-85 (2011).
9. Mendell, J.R. et al. Single-dose gene-replacement therapy for spinal muscular atrophy. *New England Journal of Medicine* **377**, 1713-1722 (2017).
10. Crudele, J.M. & Chamberlain, J.S. AAV-based gene therapies for the muscular dystrophies. *Human molecular genetics* **28**, R102-R107 (2019).
11. Barkats, M. SMA: from gene discovery to gene therapy. *médecine/sciences* **36**, 137-140 (2020).
12. Corti, M. et al. Safety of Intradiaphragmatic Delivery of Adeno-Associated Virus-Mediated Alpha-Glucosidase (rAAV1-CMV-hGAA) Gene Therapy in Children Affected by Pompe Disease. *Hum Gene Ther Clin Dev* **28**, 208-218 (2017).
13. Manno, C.S. et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* **101**, 2963-72 (2003).
14. Poupiot, J. et al. Role of regulatory T cell and effector T cell exhaustion in liver-mediated transgene tolerance in muscle. *Molecular Therapy-Methods & Clinical Development* **15**, 83-100 (2019).
15. Bartolo, L. et al. Dual muscle-liver transduction imposes immune tolerance for muscle transgene engraftment despite preexisting immunity. *JCI insight* **4** (2019).

16. Colella, P. et al. AAV Gene Transfer with Tandem Promoter Design Prevents Anti-transgene Immunity and Provides Persistent Efficacy in Neonate Pompe Mice. *Mol Ther Methods Clin Dev* **12**, 85-101 (2019).
17. Kuranda, K. et al. Exposure to wild-type AAV drives distinct capsid immunity profiles in humans. *J Clin Invest* **128**, 5267-5279 (2018).
18. Fitzpatrick, Z. et al. Influence of Pre-existing Anti-capsid Neutralizing and Binding Antibodies on AAV Vector Transduction. *Mol Ther Methods Clin Dev* **9**, 119-129 (2018).
19. Leborgne, C. et al. Prevalence and long-term monitoring of humoral immunity against adeno-associated virus in Duchenne Muscular Dystrophy patients. *Cell Immunol* **342**, 103780 (2019).
20. Aronson, S.J. et al. Prevalence and Relevance of Pre-Existing Anti-Adeno-Associated Virus Immunity in the Context of Gene Therapy for Crigler-Najjar Syndrome. *Hum Gene Ther* **30**, 1297-1305 (2019).
21. Bertin, B. et al. Capsid-specific removal of circulating antibodies to adeno-associated virus vectors. *Scientific reports* **10**, 1-11 (2020).
22. Leborgne, C. et al. IgG-cleaving endopeptidase enables in vivo gene therapy in the presence of anti-AAV neutralizing antibodies. *Nature Medicine*, 1-6 (2020).
23. Meliani, A. et al. Antigen-selective modulation of AAV immunogenicity with tolerogenic rapamycin nanoparticles enables successful vector re-administration. *Nat Commun* **9**, 4098 (2018).
24. Aronson, S.J. et al. A quantitative in vitro potency assay for adeno-associated virus vectors encoding for the UGT1A1 transgene. *Molecular Therapy-Methods & Clinical Development* (2020).
25. Wang, L., Wang, H., Bell, P., McMenamin, D. & Wilson, J.M. Hepatic gene transfer in neonatal mice by adeno-associated virus serotype 8 vector. *Hum Gene Ther* **23**, 533-9 (2012).
26. Bortolussi, G. et al. Life-long correction of hyperbilirubinemia with a neonatal liver-specific AAV-mediated gene transfer in a lethal mouse model of Crigler-Najjar Syndrome. *Hum Gene Ther* **25**, 844-55 (2014).
27. Vidal, P. et al. Rescue of GSDIII Phenotype with Gene Transfer Requires Liver- and Muscle-Targeted GDE Expression. *Mol Ther* **26**, 890-901 (2018).
28. Meliani, A. et al. Enhanced liver gene transfer and evasion of preexisting humoral immunity with exosome-enveloped AAV vectors. *Blood Adv* **1**, 2019-2031 (2017).
29. Barbon, E. et al. Single-domain antibodies targeting antithrombin reduce bleeding in hemophilic mice with or without inhibitors. *EMBO molecular medicine* **12**, e11298 (2020).
30. De Sabbata, G. et al. in HUMAN GENE THERAPY A150-A151 (MARY ANN LIEBERT, INC 140 HUGUENOT STREET, 3RD FL, NEW ROCHELLE, NY 10801 USA, 2019).
31. Orefice, N.S. et al. Real-time monitoring of exosome enveloped-AAV spreading by endomicroscopy approach: a new tool for gene delivery in the brain. *Molecular Therapy-Methods & Clinical Development* **14**, 237-251 (2019).
32. Elmallah, M.K. et al. Sustained correction of motoneuron histopathology following intramuscular delivery of AAV in pompe mice. *Mol Ther* **22**, 702-12 (2014).
33. Falk, D.J. et al. Comparative impact of AAV and enzyme replacement therapy on respiratory and cardiac function in adult Pompe mice. *Mol Ther Methods Clin Dev* **2**, 15007 (2015).
34. DeRuisseau, L.R. et al. Neural deficits contribute to respiratory insufficiency in Pompe disease. *Proc Natl Acad Sci U S A* **106**, 9419-24 (2009).
35. Korlimarla, A., Lim, J.-A., Kishnani, P.S. & Sun, B. An emerging phenotype of central nervous system involvement in Pompe disease: from bench to bedside and beyond. *Annals of Translational Medicine* **7** (2019).
36. Colella, P. & Mingozzi, F. Gene therapy for Pompe disease: the time is now. *Human gene therapy* **30**, 1245-1262 (2019).