universität wien

MAGISTERARBEIT / MASTER'S THESIS

Titel der Magisterarbeit / Title of the Master's Thesis

„The Adeno-associated Virus Vector: Quality, Non-clinical, Clinical and Regulatory Aspects"

verfasst von / submitted by

Rafaela-Lucreţia Mihaiu

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magistra der Pharmazie (Mag. pharm.)

Wien, 2016 / Vienna 2016

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	Univ.Prof. Dipl. Ing. Dr. Manfred Ogris

Mit freundlicher Unterstützung durch Dr. Anke Hildebrandt

Abstract

Adeno-Associated Virus (AAV) showed a lot of promise as a vector for human somatic gene therapy. The success of this novel therapeutic approach is very dependent on the vector used. The creation of highly efficient and safe AAV vectors requires a deep understanding of the available scientific literature on the subject and further experimental research. This diploma thesis summarized and organized available data in four categories: Quality, Non-Clinical, Clinical aspects and Regulatory issues, as to spark interest, give an overview and facilitate entry in this research field for interested parties, because AAV mediated gene transfer has the potential to manage or cure a range of genetic diseases in the near future.

Zusammenfassung

Der Adeno-asoziierte Virus (AAV) ist ein vielversprechender Vektor für somatische Gentherapie des Menschen. Der Erfolg dieses neuen therapeutischen Ansatzes hängt stark von dem benutzten Vektor ab. Zur Herstellung sicherer und hocheffizienter AAV-Vektoren werden tiefes Verständnis der verfügbaren wissenschaftlichen Literatur zum Thema und weitere experimentelle Forschung benötigt. In dieser Diplomarbeit werden vorhandene Informationen zusammengefasst und in vier Kategorien untergliedert: Qualität, nicht-klinische-, klinische Aspekte und regulatorische Herausforderungen. Dies soll Forscher für das Thema begeistern, ihnen einen Überblick vermitteln und somit den Einstieg in dieses Forschungsfeld erleichtern, da AAV-vermittelter Gentransfer ein hohes Potential aufweist, in naher Zukunft eine Reihe an genetischen Erkrankungen zu heilen.

Acknowledgments

I would like to express my gratitude to all the people who made the creation of this paper possible, in one way or another, and to the company Baxalta, now part of Shire, for enabling this special chance to write my thesis during an internship.

I would like to thank Prof. Dr. Manfred Ogris, who accepted to supervise my thesis, offered his help whenever needed, engaged in scientific discussions and who infused me with his enthusiasm on gene therapies.

I would like to address a special “Thank You” to Dr. Anke Hildebrandt, who dedicated much of her time giving me precious input and feedback on this thesis. Your contribution has been invaluable and this paper will always remind me of our nice times at the office!

Many thanks to Felix, who helped with the citations and showed much support during the making; to Vlad, who had the initiative of the external thesis; Heike and Alex, who believed in me in times of self-doubt; and to Alex Z, my companion while editing.

Last but not least, I would like to thank my parents. I owe to you the chance of studying abroad, and it has been a wonderful journey!

Contents

1. Background

1.1. Biology of AAV

1.1.1. Virus Classification

The Adeno-associated virus (AAV) is a small, non-enveloped virus of about ~20 nm belonging to the *Parvoviridae* (parvus=small, lt.) family [1]. AAV is classified in the genus *Dependovirus*, because productive infection occurs only through co-infection of a helper virus (Herpes- or Adenovirus) [2]. Over 100 serotypes have been identified, yet AAV has not yet been associated with disease in humans [3].

1.1.2. AAV-Genome

The AAV genome consists of a single-stranded DNA construct of a length of approximately 4,7 kilobases (*kb*). The genome encodes two genes, *Rep* and *Cap*, flanked by self-complementary sequences, the inverted terminal repeats (ITRs), of approximately 150 base pairs length [2]. The ITR is the origin of replication and doubles as a primer for the DNA polymerase [2].

The *Rep* gene codes for regulatory proteins necessary for AAV's life cycle, including its replication in the presence of a helper virus [2]. The *Cap* gene codes for the three viral capsid proteins [2].

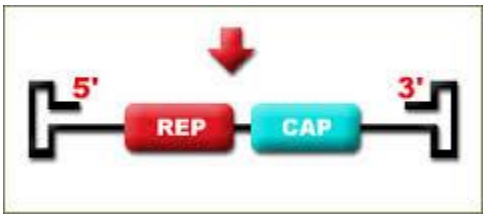

FIGURE 1: AAV GENOME WITH *REP AND* CAP *GENES FLANKED BY ITRS (SOURCE: AAVVECTORS.TRIPOD.COM [4])*

1.1.3. AAV Life Cycle

After the infection of a cell, AAV can undergo a lytic or lysogenic life cycle. The productive infection happens in the presence of a helper virus (Herpes- or Adenovirus) and leads to virion production while latency of AAV serotype 2 – in the absence of a helper virus co-infection- can happen through site-specific integration into chromosome 19 [2]. The latent genome can be rescued and lytic cycle activated through infection with Adenovirus [2]. Both stages in the AAV life cycle are modulated through genomic interactions with host- and adenoviral proteins [2].

1.2. AAV as a gene therapy vector

Gene therapy refers to the therapeutic concept, that a correct copy of a defective gene can be introduced in the host's cells, its protein is then expressed, and thus lead to either milder symptoms or complete correction of the genetic defect [5]. To reach this goal, some non-viral gene transfer methods have been tested out and used (e.g. gene gun) but most commonly viral vectors are used because of virus' natural ability to introduce their genome in the host cell [6]. The Adeno-associated virus' lack of pathogenicity, persistence of its genetic material and the many serotypes have made AAV interesting for researchers in the field of gene therapy based on a viral vector [2].

1.2.1. Manufacturing

Most commonly, AAV is produced through a method without helper virus, the triple plasmid transfection method, where human-derived HEK293 cells are transfected with three plasmids: 1) plasmid containing the *Rep* and *Cap* genes, 2) recombinant vector genome plasmid and 3) helper plasmid with adenoviral genes [7]. This way, the creation of any serotype is possible by using the appropriate *Rep/Cap* plasmid [7].

The HEK293 cells are grown in suspension and purified by ultracentrifugation and ion exchange chromatography [7]. Currently there are manufacturers producing according to GMPs available for the production of AAV vectors of serotype 1-6,8 and 9, yielding around 10^5 vector genomes (vg)/cell [7].

1.2.2. Rational design of capsids

Over 100 AAV serotypes have been identified [2], with different capsid structures, different receptors used for cell binding and internalization and, as a result, different tropisms (tissue preference) [8].

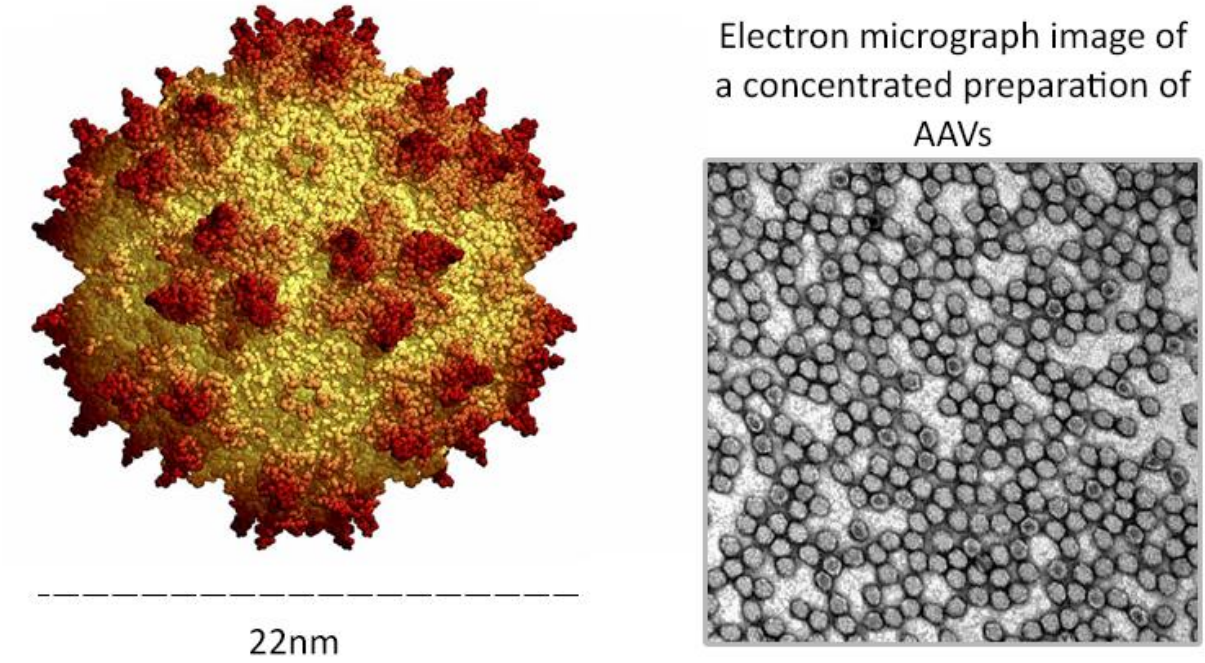

FIGURE 2: THE AAV CAPSID AND AN ELECTRON MICROGRAPH IMAGE OF AN AAV PREPARATION (SOURCE: *WWW.ABMGOOD.COM* [9]).

Considering the fact, that a segment of the population has pre-existing AAV antibodies [8], the success of the therapy being dependent on the avoidance of the immune clearance of the transduced cells, and that the clinical efficacy can be increased by minimizing off-target transduction, improvements in the field of rational design of capsids and directed evolution are currently being investigated [8].

2. Quality

2.1. General Considerations

In this chapter I will touch on the complex subject of quality control of AAV as a clinical-grade vector in gene therapy. There are guidelines [10] and current Good Manufacturing Practice (cGMP) [11] in place specifically addressing biologic products, so as to ensure a consistent purity, potency and safety of the investigational biologic product. These are comprehensive procedural controls originating from a deep understanding of biologics manufacturing, purification and characterization [12] with the scope of providing a safe and homogenous medicinal product, regardless of the manufacturing facility, methods, and etcetera.

The European Pharmacopeia, 8th edition, has a small section on "Adeno-associated-virus vectors for human use" in the 5.14 monograph: Gene transfer medicinal products for human use, which provides a general overview on AAVV considerations, also on quality, from the production to the labelling step of the manufacturing process [13].

The quality of the AAV vector product begins with the raw materials used during the production process, including components and reagents. These must show a constant high quality and sterility, and be certified [12].

The process of manufacturing and purifying the vector per se must be optimized and appropriate for large-scale production. In-process controls must be set accordingly, accompanying key production steps and the manufacturing environment must be ensured aseptic and tested, in order to prevent product contamination with pathogens [12].

The final drug product has its own aspects to take into consideration when clinical grade quality is sought after, like a stable formulation and appropriate storage conditions [12].

Figure 3 provides an example for a quality control testing strategy for clinical grade AAVV in gene therapy. It shows the well-defined vector manufacturing steps, using certified materials, as well as possible quality control tests at different stages of the manufacturing process.

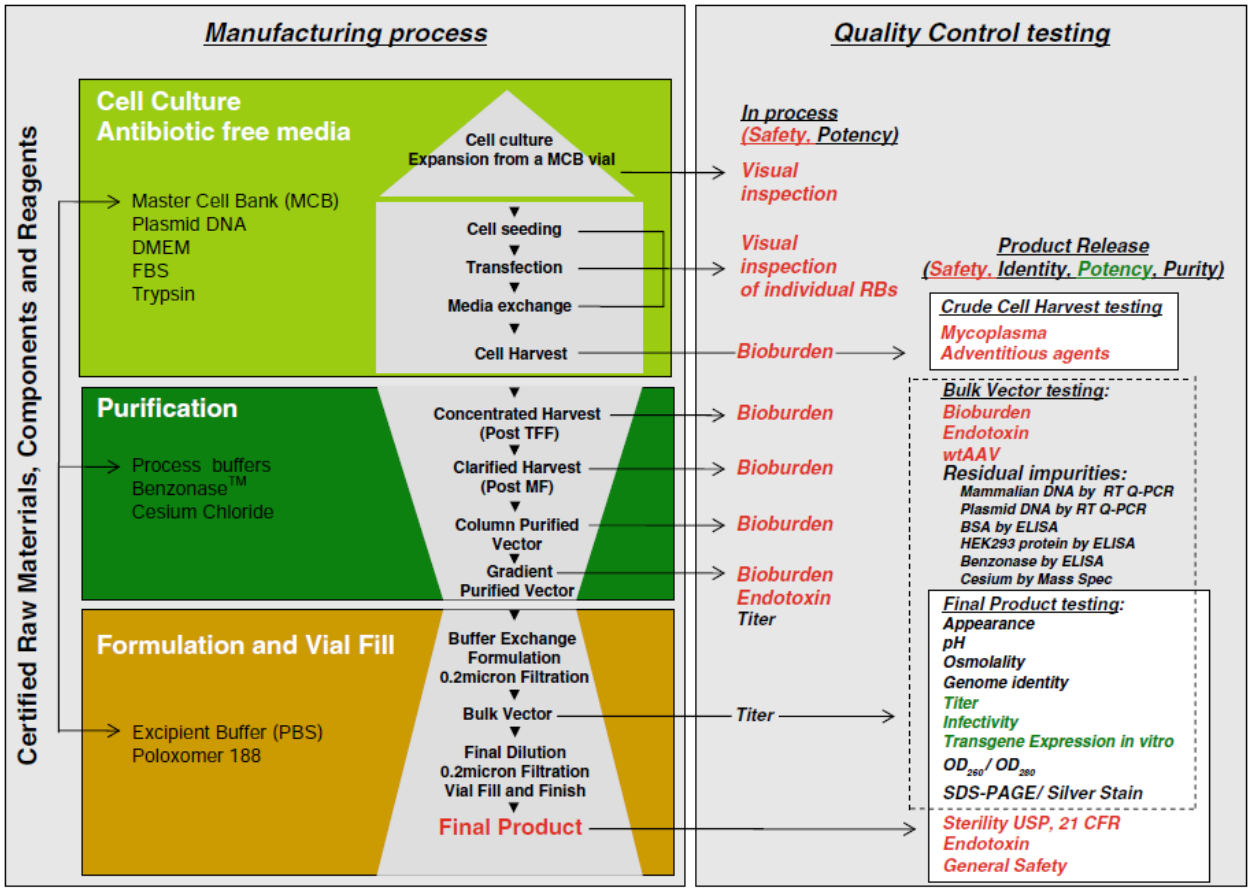

FIGURE 3. AN EXAMPLE FOR A QUALITY CONTROL TESTING STRATEGY FOR CLINICAL GRADE AAVV (SOURCE: MERTEN, OTTO-WILHELM, AND AL-RUBEAI, MOHAMED. *VIRAL VECTORS FOR GENE THERAPY: METHODS AND PROTOCOLS* [12]).

Quality Control (QC) Testing, as defined by the World Health Organization (WHO), is the sum of analytical procedures [performed in a qualified/specialized laboratory], which ensure the identity and purity of a particular drug [14]. Predefined specifications are set to guarantee this and must be respected, in order to have a well-characterized vector, as this is a must for the clinical trial application and subsequently the marketing authorization application [10]. For this to happen,

the regulating authorities must agree to the set specifications; these shouldn't be too wide, as this might pose a threat to patient safety and also shouldn't be too narrow, as this would mean a greater struggle to get lots approved and released for the company producing the therapy. Specifications are quality standards that are usually undisclosed by the producing company and the regulatory authorities, nonetheless **Error! Reference source not found.** shows different parameters used to assess product quality and suggested plausible specifications for an AAVV based gene therapeutic product.

TABLE 1 PARAMETERS USED TO ASSESS AAVV QUALITY AND SUGGESTED SPECIFICATIONS (SOURCE: MERTEN, OTTO-WILHELM, AND AL-RUBEAI, MOHAMED. *VIRAL VECTORS FOR GENE THERAPY: METHODS AND PROTOCOLS* [12])

Test	Method	Specification
Harvest		
Safety: Viral contaminants	21CFR, in vitro assay	Not detected
Safety: Mycoplasmas	21CFR	Negative
Bulk drug substance		
Appearance	Visual inspection	Clear, colorless solution
pH	Potentiometry	7.3 ± 0.5
Osmolality	Osmometry	300–400 mOsm/kg
Potency: VG titer	Q-PCR	Product specific
Potency: Infectivity	Limiting dilution in C12 cells[59]	<100 vg/IU
Potency: In vitro expression	In vitro transduction/ELISA	Product specific
Purity: Protein	SDS-PAGE	Comparable to Reference
Purity: OD_{260}/OD_{280}	Spectrophotometry[78]	≥1.2
Purity: Residual host cell DNA	Q-PCR	<10 ng/dose
Purity: Residual plasmid DNA	Q-PCR	<100 pg/10^9 vg
Purity: Residual BSA	ELISA	Report result
Purity: Residual HEK293	ELISA	Report result
Purity: Residual Benzonase™	ELISA	<1.0 pg/10^9 vg
Purity: Residual cesium	Mass spectrometry	< 0.1 µg/10^9 vg
Safety: Endotoxin	LAL	<10 EU/mL
Safety: Sterility	Bioburden	Negative
Safety: wt AAV	Infectious Center Assay	<1 rcAAV/10^8 vg
Final product		
Appearance	Visual inspection	Clear, colorless solution
pH	Potentiometry	7.3 ± 0.5
Osmolality	Osmometry	300–400 mOsm/kg
Vector genome identity	DNA sequencing	Matches Reference
Potency: VG titer	Q-PCR	Product specific
Potency: Infectivity	Limiting dilution in C12 cells[59]	<100 vg/IU
Potency: In vitro expression	In vitro transduction/ELISA	Product specific
Purity: Protein	SDS-PAGE	Comparable to Reference
Safety: Sterility <USP>, 21CFR	21CFR	Negative
Bacteria and fungistatic activity	21CFR	No B and F activity
Safety: Endotoxin	LAL	<10 EU/mL

For more information on regulatory guidelines please refer to chapter Regulatory aspects.

2.2. Safety aspects

Since some AAV gene therapies are delivered intravenously [15], they obey to the pharmacopeial set of rules for parenteral medication, which include strict limits on microbial contamination [16]. Thus, quality tests must be performed in order to ensure the safety of the product, like assays for endotoxins, adventitious viral agents (AVA) or mycoplasma, to name a few [12]. Acceptance criteria usually depend on the planned route of administration, intravenous formulations having different requirements than e.g. delivery to the lung with a nebulizer, but aseptic conditions must be ensured throughout the manufacturing process and sterility testing must be performed before batch release, on the final drug product [16].

Generally speaking, the companies developing such products should always utilize an appropriate, sensitive and validated analytical method [12], so in the following I will present possible analytical options which could be used as in-process controls in regard to product safety.

We start chronologically, with the harvest, when it is indicated to test for mycoplasma [17] and viral contaminants [12].

2.2.1. Mycoplasma

Mycoplasmas are dependent of host cellular nutrients and represent a danger to cell lines used for the production of biological and pharmaceutical products. They can cause bacterial infections, especially in the pediatric, geriatric or immunocompromised population [17]. EMA's paper "VICH GL34: Biologicals: testing for the detection of mycoplasma contamination" was released in an effort to harmonize testing procedures, these being described by the European Pharmacopoeia, the United States Code of Federal Regulations and the Japanese "Minimum requirements of biological products for animal use (2002)" [18]. This draft guideline describes two test methods, the first being an "expansion in broth culture and detection by colony formation on nutrient agar

plates" – also referred to as the "culture method", the second "expansion in cell culture and characteristic fluorescent staining of DNA (a technique capable of detecting non-cultivatable strains).", also known as the "indicator cell culture method" The testing result must be "negative" in order for the sample to be considered suitable for use [18].

2.2.2. Viral Contaminants

Tests for adventitious agents include viruses, which might contaminate biologics-producing cells and their products [19]. Because AAVVs can be produced in a cell system using helper viruses [12] and even virus-free production systems might become contaminated by unknown viral agents, it is important to determine the presence (or absence) of adventitious agents.

FDA's "International Conference on Harmonization; Guidance on Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin; Availability" publication lists some examples of virus tests to be performed at various cell levels, like in-vitro or in-vivo assays, antibody production tests or other virus specific tests [20]. In Vitro assays are carried out by inoculation into various indicator cell cultures, capable of detecting a wide range of viruses [21]. In vivo assays can be performed for example in mice or embryonated hen eggs, following a very simple principle: see if the animal gets sick by closely monitoring their health state [21]. Antibody production tests are also a method suitable for species-specific viruses; they imply the analysis of test animals' plasmatic enzymes or antibodies at a specific time point post-infection [21].

From a regulatory perspective, considering that harmonization of inter-national standards is underway but not yet completed, it is more difficult for companies to choose appropriate analytical assays, their standardization and validation being a time-consuming issue [22].The Bulk Drug Substance (BDS) should be tested for endotoxins, sterility and wild-type AAV (*wt*AAV) to ensure final product safety.

2.2.3. Endotoxin

Endotoxins are considered the major pyrogens in contaminated drugs. Endotoxins refer to large molecules, the lipopolysaccharides, on the outer membrane of gram-negative bacteria, which, if present for example in parenteral products can trigger fever, induce an inflammatory response, shock, lead to organ failure and death in humans and other animals [23]. Thus, parenteral products need to be tested for endotoxins. The United States Pharmacopeia introduced the rabbit pyrogen test (RPT) in 1942, which is an in vivo test on rabbits. After the discovery that amebocytes of the horseshoe crab clot in the presence of endotoxins the in vitro Limulus Amebocyte Lysate (LAL) test was developed [23]. This testing method has since been preferred, in vitro methods being easier to handle in a laboratory, sparing many rabbit lives and delivering results in few hours [24]. The FDA proposes a formula to correctly set the specification for endotoxins for each pharmaceutical product, starting from the set upper limit of 5 endotoxin units (EU)/kg, which is considered to be the pyrogen threshold in humans. This value then has to be divided through the maximum dose for humans per kg administered in a one hour timeframe [25]. The European Pharmacopoeia, edition 8.8, also gives the same formula for the calculation of the endotoxin limit, the threshold pyrogenic dose of endotoxin per kg divided through the maximum recommended bolus dose of product per kg and defines the threshold as 5.0 IU of endotoxin per kg of body mass for drugs delivered intravenously [26], which would apply for parenteral AAV formulations.

2.2.4. Sterility

The sterility of the BDS can be measured quantitatively through the bioburden method, also known as the microbial limit testing [27]. This testing approach can be performed through different methods, the most prominent being the membrane filtration method and the plate-count method [13]. Both rely on the cultivation of existing microorganisms in the product sample on a growth medium (e.g. Soybean-Casein Digest Agar) under specific culture conditions and then quantifying the total aerobic microbial count (TAMC) and total yeasts and mold count (TYMC) [13]. There is an *ISO* standard in place for bioburden testing, ISO 11737-1:2006, according to which it will take 7 days to get the bioburden test results back [28]. For an AAV vector

investigational product for intravenous infusion, the suggested specification for bioburden would be “negative”, since sterility is defined by the absence of microorganisms [26].

2.2.5. Wild-type AAV

Even though AAV is a replication incompetent, non-pathogenic virus, QC *wt*AAV testing is required for clinical grade AAV vector products [12], the European pharmacopoeia requiring the verification of absence of *wt*AAV using NAT (nucleic acid amplification techniques) [26]. In the “nucleic acid amplification technique” the PCR is described extensively, though the pharmacopeia states that it is allowed to use alternative methods, if these are comparable in quality. A method is, for example, an infectious center assay, as described in the “Vector characterization methods for quality control testing of recombinant Adeno-Associated viruses” article by J. Wright et al [12]. In this case, the *wt*AAV is defined as replication competent in HEK293 cells in the presence of Adenovirus, so basically one tries to find out how replication competent the recombinant AAV vector product is. In other words, the test sample is not allowed to have more *wt*AAV particles than a positive control with a defined quantity of wtAAV [12]. The EMA addresses the fact that it is undesirable for a drug product to be contaminated with impurities like wtAAV [10], though this matter is not argued or discussed any further.

In the final drug product (FDP) release tests sterility and endotoxins must be tested again, amongst others [12].

2.3. Potency

In an effort to attain uniformity between drug product lots and ensure a defined dose when administered to patients, the functional activity and concentration of AAV vectors must be determined using specific, sensitive and validated methods [12]. Vector genome (vg) titer, infectivity and in vitro expression are examples of tests performed on the BDS and FDP to demonstrate the product’s potency.

2.3.1. Concentration of vector genomes

The vector genome refers to the number of viral capsids containing DNA (as opposed to, for example, empty capsid particles). Dot-blot hybridization and quantitative polymerase chain reaction (qPCR) are frequently the dose defining assays for clinical use [29], whereby PCR is method of choice for nucleic acid quantification [30] because it is more robust and easier to standardize and to validate [12].

Even so, qPCR has been shown in studies to have a high variation of the vector genome titer prompting scientists in the field of AAVV research to search for an option with less inter-laboratory variability. One possibility is the commercial ELISA kit, which had the least variation in a series of tests performed in 16 laboratories [31]. Quantification of capsid particles, empty or full, is of importance anyways, since it has been shown, that the immune response is linked to the quantity of capsids administered [32]. However, the ELISA method is serotype-specific, and even though it is available for some prevalent serotypes (AAV2, AAV8, AAV9 …) it is not a feasible method for hybrid/chimeric serotypes until the specific antibodies become available [31].

Nonetheless, if a constant empty-to-full capsid particle ratio between product lots can be demonstrated, it is possible to measure the strength in the percentage of full capsid particles and correlate the *capsid particle (cp)* unit of measure with the *vector genomes (vg)*. This allows for a more precise and consistent dosing of the gene therapy.

2.3.2. Infectivity

The infectivity is actually a combined parameter of two processes: the delivery of the transgene to the nucleus of target cells by the vector and the subsequent transgene expression, meaning the translation of newly inserted DNA to an active protein product of interest [12]. The limiting dilution method with qPCR readout is an option presented in literature as being highly sensitive and effective, to the extent that even a single infectious event would be detected [33]. The principle of this method is that HeLa-derived cell line expressing AAV2 *rep* and *cap* genes are infected with multiple defined dilutions of AAV and Adenovirus, as a helper virus. Quantitative PCR is used subsequently, at 48 hours post-infection, to determine vector genome replication through quantification of the transgene [33].

2.3.3. *In vitro* expression

In order to test and assess the functional activity of vectors, in vitro assays must be developed. For AAV this might pose a challenge, since many phenotypes show a strong tropism. After tailoring the assay in such a way, that the cell line gets infected by the viral vector, transgene protein expression can be measured by, for example, ELISA[1] and must be shown to be dose-dependent [12] so as to correlate protein expression with vector activity. This method may be sufficient for characterization of vectors used in early phase clinical trials; late phase clinical trials and licensure require a supplementary product specific assay, which quantifies the functional activity of the therapeutic product [12].

EMA expresses the wish for the development of a bioassay such as $TCID_{50}$[2] to reflect AAVV's infectivity, based on a paper published in 2005 by Schiau [10]. In this study the biological titration of rAAV is done using a helper virus which is not capable of causing structural changes in host cells, thus reflecting the infectivity of rAAV independent of the helper virus' infectivity [34].

EMA postulates that a combination between an infectivity assay and packaged DNA quantification would produce the most accurate measure of AAVV full particle to infectivity ratio, which in turn would allow for a more precise dosing [10].

2.4. Purity

AAV based therapies strive to limit any immune response as much as possible, in order to achieve stable and long-term transgene expression, hence it is imperative that a high level of product purity is sought after, since impurities may contribute to in vivo immune responses [35]. Moreover, it has been shown, that high AAVV purity results in enhancement of transduction,

[1] The enzyme-linked immunosorbent assay (ELISA) is a test that uses antibodies and color change to identify a substance.
[2] 50% tissue culture infectivity dose

regardless of serotype and tissue, thus having implication not only in the therapy's safety but also its efficacy [36].

Purity testing is usually done on the bulk drug substance and final drug product, after the purification steps of the manufacturing, quantifying for example process impurities like residual host cell components, residual DNA from the plasmid production step and possible contamination from reagents [12]. During production there is also the risk of impurities like AAVV transgene protein product or from helper virus constituents [10].

2.4.1. Residual impurities

During the manufacturing of AAV, especially after the lysis of the cell harvest, performed in order for the newly produced virus to be set free, a purification step must take place that separates the AAV vector from the protein "soup" of lysed cells, plasmid genetic material fragments and reagents. This is shortly described in chapter 1.2.1, "Manufacturing". Any remaining particles other than AAVV are categorized as "residual impurity" and must be checked for and quantified, in order to ensure product quality. Ideally, of course, the residual impurities are absent, practically, each company developing AAVV based therapies must set their own specifications, as mentioned earlier in this chapter, because process impurities do occur and are tolerated up to a certain amount.

Residual host cell DNA and plasmid DNA is usually quantified by qPCR [26], the golden standard for nucleic acids quantification. In order to decrease the risk of the residual DNA of integrating into the treated patient's genome, endonucleases are used for cleaving genetic material into pieces that are smaller than genes (approx. 100 base pairs) [37]. Total endonuclease residue can be quantified by ELISA [12]. Other reagents used during production must be tested for except if the process has been validated to show suitable clearance [26]. Another common process impurity from reagents is cesium, cesium chloride (CsCl) being often used in a purification step. Residual cesium can be quantified by mass spectrometry [12]. If antibiotics were used during production, the residual concentration must be determined by microbiological assay or another suitable method [26].

HEK293 cell line residues and BSA (bovine serum albumin), if bovine serum is used for cell-culture vector generation, can also be quantified by ELISA.

Vector purity, or rather residual impurities, can also be quantified by spectrophotometry, following a very simple principle: if the extinction coefficient for the AAV capsid proteins and the encapsulated DNA is known, a comparison of measured sample results with the hypothetical extinction of a 100% pure product would result in a statement on the proportion of residues in the sample [12]. Another method of separating AAVV from other proteins is SDS-PAGE, the sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2.4.2. Vector impurities

Vector impurities refer to either empty viral capsids or capsids carrying wrong DNA. They represent an unnecessary increase in viral antigen load, if not removed. During production, empty AAV capsids are generated to an extent of over 50% [38]. Incorrect DNA, both from helper plasmids or from production cells, can be encapsulated into AAV and is not sensitive to nucleases [12], packaged DNA impurities accounting for 1-6% of total DNA in purified vector particles [39]. If undesired genetic material is found in the AAVV capsids, the manufacturer must quantify it and should investigate the possibility of its translation in mammalian cells [10]. Its levels can be assessed by qPCR using primers and probes [39] and translation should be investigated on a range of cell lines that reflect AAV tropism and distribution [10].

A comprehensive risk assessment is required if viral genomes containing co-packaged DNA are shown to persist in cells for an extended period, in order to demonstrate unaltered product safety [10].

Reference standard material is already available for the most prevalent AAV serotypes, like AAV2 or AAV8 [31], deposited by the Adeno-associated Virus Reference Standard Working Groups (AAV2RSWG and AAV8RSWG) on the *ATCC.org* website (https://www.lgcstandards-atcc.org/).

2.5. Stability

Stability refers to the long-term quality of the therapeutic product. Stability studies provide information on time's and environment's influence on the quality of the product [40]. It can be described through two main considerations: first of all, the determination of the shelf life of the vector product. This can be determined through a stability study by observing the product after storage under designated conditions (room temperature, humidity, light, etc.) and a certain amount of time. The shelf life must be determined not only for the final drug product (FDP) but also for the drug substance and intermediates subjected to storage [41]. Secondly, it must be proven that the vector product doesn't interact with the immediate packaging material. By this is meant any type of reactivity and/or absorbance processes that could modify or harm the active substance [40].

EMA proposes a stability protocol for gene therapies consisting of appearance, pH, genomic titer, infectious titer, in vitro potency and purity by SDS-PAGE [41]. These parameters have been discussed in the chapter Purity and Potency, except for appearance and pH. The pH of the gene therapy has to have a physiological value, if intended for parenteral use, since the buffer capacity of blood is limited between 6.6-7.4 [42]. Also when used as a parenteral, the appearance is analyzed through visual inspection and the specification is "clear and practically free from particles", as stated in the U.S. and European Pharmacopeia for parenteral formulations [43].

The medicine Glybera, marketed by UniQure pharmaceuticals and which uses an AAV as a vector, originally received a negative opinion by both the CAT (Committee for Advanced Therapies) and the CHMP (Committee for Human Medicinal products), who were evaluating the therapy on behalf of the European Medicines Agency (EMA). Their main objections were in the fields of quality, but the company addressed the concerns and managed to obtain a marketing authorization for the first gene therapy in the western world. Specifically, the authorities expressed six major objections, one of them regarding the structure and the other five in the area of „quality" [44].

The company proposed to eliminate the assay quantifying replication competent AAV (rcAAV), since their initial results on the tested batches demonstrated a lack of rcAAV but since this was a major objection they agreed to reintroduce routine rcAAV assays and to develop assays with greater sensitivity [44].

The assessors expressed two objections regarding Glybera's impurity profile, the first being the residual baculovirus DNA, which the producing company solved by presenting data supporting the fact that these impurities do not affect the product's safety profile, and secondly the general amount of impurities co-administered, which prompted the company to commit to develop more sensitive assays post-opinion and to introduce an extra virus inactivation step for the baculovirus used in the production process.

The developing company also had to revise its potency specifications and re-think its infectious titer assay, so as to clearly make a statement regarding the particle infectivity and, in consequence, of its potency [44].

As effect to the solving of all six major objections the two committees evaluating the medicine re-examined their recommendation for Glybera, the CAT changing it to "positive" and the CHMP backing CAT up in the decision to recommend approval of the medicine to the European Commission [45]. This resulted in pharmaceutical history: the granting of a marketing authorization for alipogene tiparvovec (Glybera, UniQure) [45] which shows that with a detailed enough care for quality aspects, a gene therapy based on AAVV can find its way to the patients.

All, which is implied when stating "this is a high quality product", from the establishment of quality systems to the manufacturing facility and its equipment, the manufacturing methods and the thorough product characterization, is done in order to guarantee a safe, homogenous and carefree product for the patients. Being a novel therapeutic approach, AAVV gene therapies still have evolving quality control methods, based on gathered expertise and on new analytical technologies emerging, all with the purpose of improving the drug's safety profile and delivering the quality promise to all patients opting for an AAV therapy. But, even after extensive quality considerations, each drug must first go through non-clinical testing before it can reach testing in humans. Why this step is imperative and some examples of breakthrough AAVV preclinical gene therapy research is addressed in the next chapter, „Non-Clinical".

3. Non-clinical aspects

3.1. Introduction

The importance of preclinical studies is undebated: before testing any substance on humans, we must first learn about its pharmacodynamics and pharmacokinetic behavior and toxicological attributes. An estimation of a starting dose for first-in-human use is also necessary, so as to avoid unwanted toxic effects.

After 25 years of basic AAV research, the first attempt to treat a human disease was the *in vitro* A gamma-globin gene transfer by rAAV [46]. The Samulski lab managed to show that rAAV encoding the human A gamma-globin could be stably introduced into human erythroid cells and the expression was equivalent to that of the native gene [47]. Shortly thereafter the first *in vivo* administration of AAV took place in rabbits, the experiment being a success: the scientists could show efficient transduction of the AAV-encoded Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene in lung tissue and CFTR protein was quantifiable even 6 months post administration [48].

The first study demonstrating long-term transduction in an animal model, after other vectors failed to do so because of immune clearance, was done on mice with rAAV and was able to demonstrate that AAV-transduced cells do not spark an adaptive immune clearance [46]. The first *in vivo* biodistribution studies with fluorescently marked rAAVs not only showed the first clues for viral tropism, but delivered a deeper understanding of AAV biology: their genomes appear in episomal form and not integrated [46].

Characteristics like immunogenicity and tropism, both researched in animal models, come with distinct sets of advantages but also disadvantages/dangers. In the following I will discuss some special considerations for the AAVVs in the non-clinical research phase.

3.2. Choice of animal model

Adeno-associated virus is a species specific virus [10] which means that in order to get clinically relevant data, special attention is required for the selection of a suitable animal model.

Differences may occur in biodistribution, for example, because of differences in more specialized mechanisms, like the structure of the receptor used for the virus's entry in the cell [49; 50]. In the quest to gather as much information as possible about what the AAVV does to a cell and in an organism before going into the human body, using more than one animal model might be beneficial.

Studies with Glybera, a gene therapy indicated for Lipoprotein Lipase Deficiency (LPLD) which got regulatory approval in EU, have been performed on LPL-deficient mice and cats, to demonstrate proof of principle for the therapy. Both disease animal models have in common the high plasmatic triglyceride levels with humans suffering from this condition [44].

AAV1 carrying the therapeutic LPL gene was tested in mice and showed promising results, like a reduction of up to 99,2% in plasma triglyceride concentration and residual protein activity 52 weeks after administration [44]. Another finding was the dose-related correspondence of LPL activity and protein content, which has implications for the first-in-human dose calculation, so as to not administer a futile dose to patients. Injected muscle tissues showed long term expression of quantifiable human LPL activity over a year, with immunohistochemical analyses confirming the expression of LPL at the outer surface of the muscle [44]. The researchers also tested whether there is a difference in transgene expression, when administering the same dose using 4 or 36 injections, coming to the conclusion that this plays no role in mice. Furthermore, after the administration of a lipid challenge, treated mice recovered more quickly and efficiently from the high plasma triglycerides than untreated mice [44]. Equally important was the finding that administration of a second dose did not yield any results, which was attributed to the AAV1 capsid neutralizing antibodies [44]. Such a conclusion is not achievable *in vitro*, which just goes to show that meaningful *in vivo* models are essential for AAV research.

The first rAAV study in large animals was performed on hemophiliac dogs [46]. The research group injected rAAV encoding FIX in the dogs' muscles and was able to show FIX expression for over ten weeks [51]. The safety data gathered in this study encouraged the development of gene therapies for humans [46].

A study performed on hemophiliac dogs of an AAV2/8 mediated factor IX gene transfer in hepatocytes also represents a great milestone in gene therapy research. The study performed by

Wang et al. demonstrates sustained correction of hemophilia B in a large mammal, which is a proof-of-concept precursor for clinical i.v. application [52]. Another breakthrough was recently achieved on dogs; a group of researchers submitted the results of their studies on FVII deficient dogs at the end of 2015, demonstrating that an AAV mediated expression of zymogen FVII can lead to therapeutic FVII levels for over a year [53].

Large animals are represented in the preclinical trials also by non-human primates [54]. Additional research on monkeys showed a low toxicity for AAV gene therapy [54], which in turn sparks more interest for further development of AAV therapies for human conditions.

Under special conditions, where the researchers can justify their approach, testing on animals with a species-specific, different AAV serotype than that used in humans is accepted, so as to better mimic biodistribution patterns and/or pre-existing immunity to the capsid particles [10]. In addition to this flexibility, authorities also accept the use of species-specific transgenes in pre- and clinical trials [10]. This is based on the rationale that human genes code for human proteins, which might spark an immune response in the animal and this, in turn, clears transduced cells [10] perhaps leading to wrong conclusions about the therapy's efficacy and immunogenicity. If the case should arise, that the applicant wants to support a clinical trial application with data collected with species-specific serotype and/or transgene(s), it is suggested that a scientific advice be sought before proceeding with the pre-clinical testing [10]. More on scientific advice on gene therapies in Regulatory aspects.

3.3. Vector persistence

Not long ago, in the last decade of the 50-year-old AAVV research history, studies on animals have been published which come to the conclusion that adeno-associated vectors might spark insertional mutagenesis [55; 56]. This hypothesis is still under debate, yet it raises certain concerns [10] for researchers and study participants alike.

Many non-clinical and clinical trials have shown a good safety profile for the AAV vector [57; 52], with the most severe side effect being a transient hepatitis [58], yet neonatal mice administered AAV2 show an increased incidence of hepatocellular carcinoma (HCC) [55]. Based on the fact that AAV genome fragments were found integrated in a site near a microRNA (miRNA) cluster, the

authors conclude that the oncogenic mechanism is based on insertional mutagenesis [55]. Figure 4 shows the hypothesized mechanism of insertional mutagenesis.

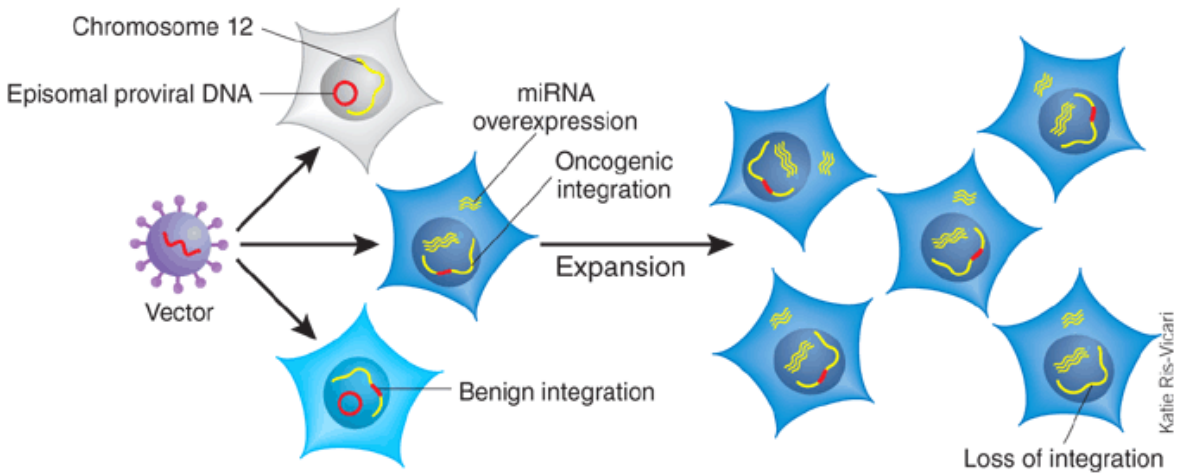

FIGURE 4: FATE OF AAV VECTOR GENETIC MATERIAL IN THE HOST CELL, INCLUDING THE HYPOTHETICAL MECHANISM LEADING TO ONCOGENESIS THROUGH INTEGRATION (SOURCE: KAY, MARK A. "AAV VECTORS AND TUMORIGENICITY." [56].)

This theory is, however, frowned upon, firstly because AAVV's genome has a mostly episomal persistence, with only 0.1-1% transduction events [59], and secondly because two independent laboratories were able to show that the mechanism responsible for the oncogenesis was not insertional mutagenesis [56]. The idea, that AAVV proviral DNA integrates preferentially near or in transcriptionally active genes is also widely debated [59].

Nonetheless, authorities feel compelled to prompt researchers to include *in vitro* and/or *in vivo* studies on whether episomal or integrated long-term transgene expression is to be expected [10]. In the event of integration, further studies might shed light on whether the integration happens preferentially in some tissues, whether it occurs in transcriptionally active loci on the chromosome and whether or not it shows potential for activation of nearby genes [10].

The "Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal products" (EMEA/CHMP/GTWP/12459/2006) encourages the assessment of vector persistence and shedding in animal models. AAV vector persistence is observed mostly episomally but not only in target tissue, since AAV's tropism is not exclusive [10]. Therefore, it is

rational to observe the level of episomal and ectopic gene expression and to adapt the duration of the non-clinical studies evaluating vector persistence to the rate of cell renewal [10].

3.4. Tissue tropism

Different AAV serotypes have been observed to have a preference for transducing one or more specific types of tissue [8]. Natural evolution has led to the creation of different serotypes with different tropisms, like AAV1 having a preference for transducing muscle, AAV8 for the liver and AAV6 for the heart [8].

Apart from the natural AAV serotypes scientists have used advanced viral engineering techniques to produce new serotypes. These "hybrid" AAVVs can be modeled in such a way as to bring therapeutic advantages. Firstly, vectors with capsid particles which differ from the wild-type may evade an immune response [46] and in turn increase the therapy's efficacy. Secondly, rational design of the AAV capsid might help improve its toxicity profile [46] and, lastly, optimize vector-host-interactions [8]. This final consideration refers to more than one option: tropism can be enhanced in target-cells, decreased ("de-targeting") in off-target cells [46] or even allow non-permissive cells to be transduced [8].

Research teams tailoring AAV for clinical application mostly focus on ways through which they can modify the viral capsid and two main approaches have been applied: knowledge-based rational and directed evolution [8]. Both ways use firstly *ex vivo* and subsequently *in vivo* methods to test the resulting mutants for tropism and transduction efficacy, before it is tested clinically [8]. Nonetheless, it is imperative to keep in mind that AAV can have a different biodistribution pattern cross-species because of potentially different receptor affinities [8]. Authorities encourage a cautious approach for the translation of non-clinical tropism data to humans [10].

3.5. Reactivation of productive infection

In 1996 a study was published which investigated the rescue of AAV vectors from rhesus macaques who were infected at a later point with the corresponding wild-type (*wt*) virus [60]. The researchers applied the AAV-CFTR vector to the respiratory nose and lung epithelium of the

test-monkeys and firstly co-infected harvested cells *ex vivo* with *wt*AAV and Adenovirus (Ad). Confirmed episomal vector DNA was rescued in this manner [60]. *In vivo* the co-infection with Ad and *wt*AAV took place either before or after the gene therapy and subsequent shedding studies showed that AAV-CFTR could be rescued in both scenarios [60]. This warns about the possibility of viral re-activation after the patients get infected with a *wt* and a helper virus and this possibility should be investigated in non-clinical studies, according to authorities [10]. This 1996 study conducted by Afione et al. [60] is given as an example for such a non-clinical study [10].

Additionally, in the eventuality of a clinical investigational protocol which includes associated treatment (e.g. immunosuppressive agents or chemotherapy) administered concomitantly with the AAV investigational product, an increase in the risk of viral reactivation should be addressed during pre-clinical evaluation [10].

3.6. Germ-line transmission

Germ-line transmission of vector DNA raises ethical and safety concerns [61]. Laws in EU prohibit clinical trials of gene therapies which modify the subject's germline genetic identity; therefor pre-clinical studies addressing this topic must be performed in order to evaluate a new medicine's potential for vertical transmission of vector genetic material [61].

AAV DNA has been found in mice and rats' gonadal DNA, though not in rabbit and dog, following the administration of rAAV2 coding for the coagulation factor IX [62]. The detection method was PCR and the authors found a correlation between vector dose and the likelihood of germ line transmission [62]. Their conclusion was, nonetheless, that "the risk of inadvertent germline transmission of vector sequences following IM or hepatic artery injection of AAV-2 vectors is extremely low" [62].

In addition, in clinical trials using rAAVVs, germline transmission has been confirmed in humans but only transiently [32]. The detection by PCR of vector DNA in motile sperm initially worried the authors of this study, but were nonetheless relieved when it was proven to be only transient. They correlated the age of the participants with the clearance of sperm, younger subjects clearing more quickly that older patients [32]. During this time, participants were recommended that they bank sperm before enrollment and that they use barrier methods of contraception until semen samples came back negative for vector sequences [32]. In addition to this, experimental data in

humans and mammals shows that the vector detected in the seminal fluid does not transduce the motile sperm cells [62].

3.7. Environment risk considerations

The European regulatory framework requires an environmental risk assessment as a part of the therapy's marketing authorization application [63] which has special requirements for GMOs like the AAVVs. This risk assessment has the scope of identifying any risk that the release of the GMO into the environment might have on human (non-patient) health and that of the environment [63]. This is tightly connected to vector shedding, which data suggests is dependent on the dose and route of administration [10]. Any vector shedding data can be used in the environmental risk assessment [10], so companies developing gene therapies can choose to investigate the extent of infectious particles released into the environment also in animal models. Yet shedding studies in the pre-clinical phase are not obligatory, as for example Glybera only presented shedding results from patients enrolled in the clinical trials [44]; Nonetheless, using animals in shedding studies also has the advantage of better designing these studies in humans, e.g. for a better estimation of the duration of follow-up necessary or the determination of sampling frequency. Non-clinical shedding studies can therefore justify less extensive shedding studies in humans [10].

While an effort to reduce the number of animals used in non-clinical testing in the pharmaceutical industry is well underway, studying new substances or new therapeutic concepts *in vitro, in vivo* and in similar organisms is essential for the safety of the people who enroll in clinical trials. After several successful pre-clinical AAV studies [48; 51] scientist started administering rAAVVs to patients. The hopes and disillusions of this new chapter on clinical therapeutic applicability are discussed further in "3. Clinical aspects".

4. Clinical aspects

4.1. General

Recombinant vectors based on the adeno-associated virus (AAV) are currently in use in a multitude of clinical trials, even in children [64], with reports of benefit for patients suffering of, for example, hemophilia [57] or inherited blindness [65]. Glybera, the first gene therapy authorized by the European Commission in 2012 [66], is an AAV1 viral vector which delivers a copy of the human lipoprotein lipase (LPL) gene to muscle cells in patients suffering of lipoprotein lipase deficiency (LPLD), thus consolidating AAV's position as the top runner viral vector for use in the clinic.

Qualities previously demonstrated in non-clinical trials like long-lasting transgene expression or low immunogenicity [52] have sparked interest in the use of AAV vectors in gene therapies, though it has been shown experimentally that not all animal data can be transferred successfully to human models [67]. Competent authorities stress this fact in different guidelines, for further reference see chapter Regulatory aspects.

Initial targets for gene therapy included monogenic diseases, in which either an important cellular product is missing or becomes non-functional through incorrect genetic information [2]. A success in treating/curing monogenic diseases through gene therapy would open up possibilities in treating gradually more complex genetic disorders. But until then, the gene therapeutic process must be profoundly understood and more data gathered, as to unfold its complete potential. In this chapter I will take a retrospective look at clinical trials using an AAV vector for gene therapy in humans, focusing especially on the treatment of hemophilia and treatment of Leber congenital amaurosis (LCA).

4.2. Clinical

Clinical trials using AAV as a vector account for 6,7% of gene therapy clinical trials, the most commonly used capsid is the Adenovirus, with 21,7%(n=506) [68].

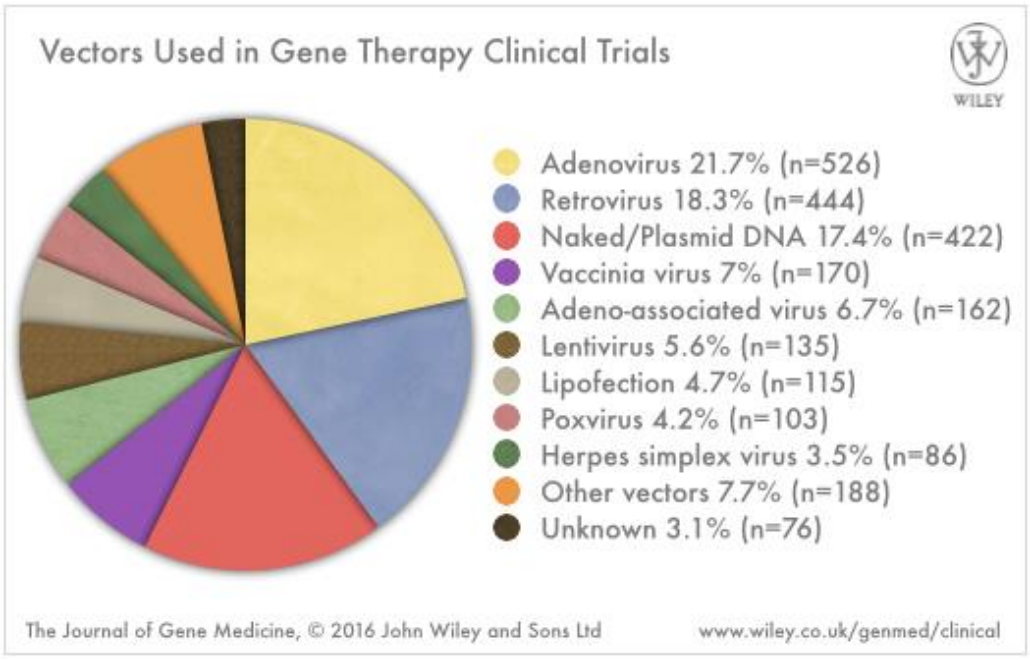

FIGURE 5: VECTORS USED IN GENE THERAPY CLINICAL TRIALS (IN PERCENT) (SOURCE: *HTTP://WWW.ABEDIA.COM/WILEY/* [68])

Few AAV clinical studies reach phase III, as is true about investigational medicinal products in general, even fewer receiving a marketing authorization. But currently (status as of June 2016) 8 phase III AAV clinical studies are being undertaken: one for a therapy against Leber Congenital Amaurosis and seven targeting prostate cancer. All of the prostate cancer clinical trials, opened 2004-2006, use an AAV to insert the gene for Granulocyte-macrophage colony stimulating factor (GM-CSF) in the tumoral tissue [68].

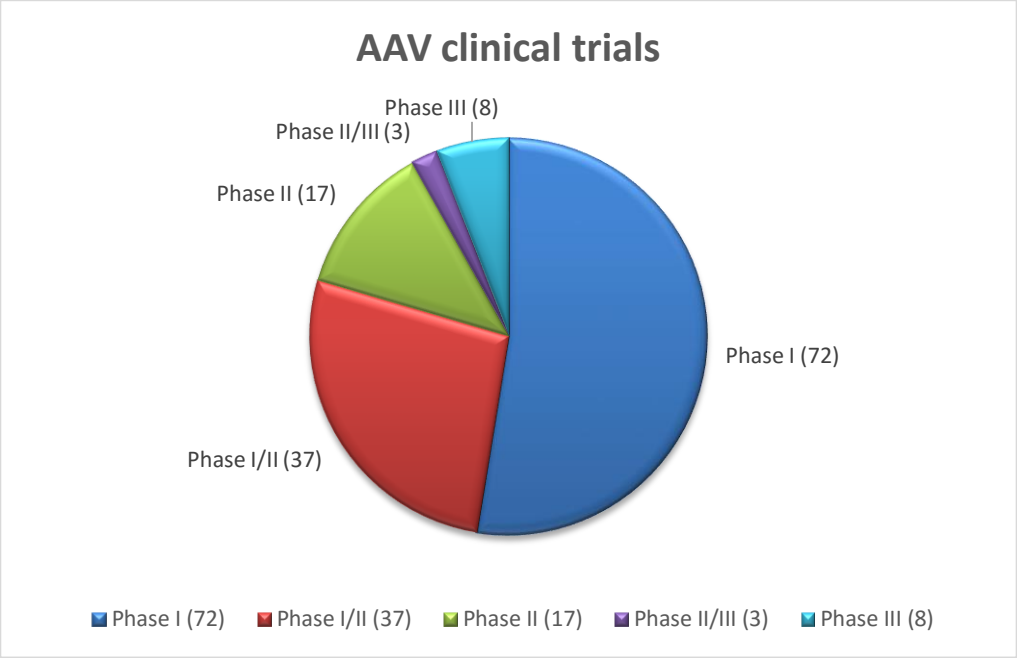

FIGURE 6: DISTRIBUTION OF CURRENT AAV CLINICAL TRIALS IN DIFFERENT TESTING PHASES [68]

One of the earliest studies for AAVV gene therapy was in patients suffering of **cystic fibrosis (CF)**, an autosomal recessive disease, which has no cure. Here, the CF transmembrane regulator (CFTR) is inactivated by a mutation that leads to the accumulation of thick secretions in the lungs. This leads, together with the loss of epithelial ciliary activity, to an increased incidence of pulmonary infections [2], pulmonary problems being responsible for death of 80% of people with CF [69]. CFTR knock out mice are available, but they have proven not to be of use in non-clinical models because they develop only intestinal defects rather than lung infections [70]. Over ten protocols were approved for phase I and phase II clinical trials using AAV vector [2]. In this case, the delivery into the lung could be performed locally, by aerosol or bronchoscope. The primary observation was that of a low immune response and of lack of measurable toxicity [2]. However, there was no statistically significant improvement of the lung function, the parameter used to describe the efficacy of the therapy. Also, the airway delivery system has proven to not be optimal, because of the rapid, regular shedding of the respiratory epithelium. Additionally, the mucus layer covering the targeted cells might impede gene delivery by viral vector systems [70]. The studies on gene therapy against cystic fibrosis had the clinical outcome of a safe gene transfer to nasal sinus and bronchio-epithelium with a moderate correction of electrophysiological defect and

hyperinflammatory responses and an overall statistically non-significant improvement in lung function in patients. Nonetheless, the limitations seem to be the short duration of the biological effect and the failure to stably transduce target cells [71; 72]. These investigations paved the way for subsequent administration to other tissues by revealing much of the safety of the vector in humans. For example, one of these studies, Flotte's 2003 study "Phase I trial of intranasal and endobronchial administration of a recombinant adeno-associated virus serotype 2 (rAAV2)-CFTR vector in adult cystic fibrosis patients: a two-part clinical study." [73], demonstrated gene transfer to bronchial and nasal tissues after a local application of tgAAVCF to nose and to the lower lung lobe. The DNA transfer was measured with DNA PCR 30 days after vector administration and observed only in the two highest dosing cohorts (highest dose of 2×10^{12} DNase resistant particles (DRP)), in the range of 0.002 to 0.5 copies/cell, which indicates an efficient DNA transfer. However, measurements of transepithelial potential difference[3] did not indicate to a clear physiologic effect. This might be because of physiological barriers, the authors conclude. Nonetheless results are promising, the investigators being able to demonstrate gene transfer through aerosol delivery of genes packed into an AAV capsid.

Another field of interest on the applicability of gene therapy mediated by an AAVV is the treatment of **retinal diseases**. This represents also a special case since the eye is a so-called "immune privileged space". This is a term coined by Sir Peter Medawar to describe the lack of an immune response against grafts placed into the ocular microenvironment. This happens because of an anatomical feature, a blood barrier, and a lack of direct lymphatic drainage [74], features which would prove to be key in the success of gene therapies for retinal diseases because of the lack of an immune response, as well against the vector capsid as well against the transgene or the transgene product. Other features, which make the eye an ideal candidate, are its small size and subsequent small dose of vector necessary, and its compartmentalized structure, which permits the specific targeting of various ocular tissues. As the retinal cells do not usually divide, it is possible to use non-integrating vectors and still obtain sustained transgene expression [65]

[3] Patients suffering of CF have a more negative transepithelial potential (measured between the surface of the respiratory epithelium and the interstitial fluid). This happens due to a Na^+ hyperabsorption in the cell due to the blocked Cl^- secretion.

and another feature which comes with a pair of eyes is the fact, that the contralateral eye can serve as an internal control, making it easier to evaluate study outcomes.

The mammalian retina is composed of three layers of neuronal cells, the photoreceptor cells being located in the deepest layer. A layer of post-mitotic cells called retinal pigmented epithelium (RPE) is located right behind the retina and provides vitamins, nutrients, oxygen and gro*wt*h hormones to the photoreceptor cells [70].Photoreceptors and RPE are tightly connected through an extracellular matrix, hence, if the RPE malfunctions because of mutations of its expressed genes it can lead to degeneration of photoreceptors [70]. Most common forms of retinal diseases include glaucoma or diabetic retinopathy but there are also a large number of genetic retinal defects including some that can lead to blindness, which could be cured by gene therapy.

4.2.1 Leber congenital amaurosis

A very severe form of inherited retinal degeneration is the Leber Congenital Amaurosis (LCA). Patients suffering of LCA have very severe loss of vision and nystagmus starting in early childhood. The disease progresses usually to blindness in the 3rd or 4th decade of life [75]. One form of LCA is caused by a defective gene, the RPE65, which codes for a protein needed for the normal function of the RPE layer, 11-cis-retinal [75]. In the following, I will present two gene therapy clinical trials performed on patients with a mutational defect in the RPE65 gene.

In October 2008 the study "Treatment of Leber Congenital Amaurosis Due to *RPE65* Mutations by Ocular Subretinal Injection of Adeno-Associated Virus Gene Vector: Short-Term Results of a Phase I Trial" [76] by W. Hauswirth, T. Aleman et al. was published in the Human Gene Therapy Journal. The central question of the paper is whether a rAAV serotype 2 vector carrying the human RPE65 gene (rAAV2-CB-h*RPE65*) is safe and efficacious in three test subjects. The study has been initiated after pre-clinical models and early results of two clinical trials Maguire et al. (2008) [77] and Bainbridge et al. (2008) [78] showed great promise. In these two clinical trials, both performed on 3 adults, the results and interpretations have been different, thus leading investigators to performing another trial, also with three adults, as to gain more solid data.

The three young adult subjects enrolled, aged 21 to 24, had a clinical diagnosis of LCA with the RPE65 gene mutation. The clinical trial took place at Scheie Eye Institute of the University of Pennsylvania and the University of Florida/Shands Children's hospital (Gainesville, FL), where the test subjects got the vectors injected into the subretinal space of their eye with the worse visual function. The intervention took place under local anesthesia and the sclerectomy sites and conjunctiva was sutured where incisions were made. Subconjunctival and topical antibiotics and steroids were administered for up to 20 days post-surgery.

The main outcomes in terms of immune response and vector biodistribution are, that there was no report of statistically significant increase in circulating anti AAV2 antibodies 14 and 90 days post treatment. T-cell immune response to AAV2 capsid was not positive in any of the three subjects and in biodistribution studies none of the patients showed AAV2 in peripheral blood and no vector genomes were detected at any time point anywhere in bodily fluids up to 14 days post-administration.

In terms of improvement of vision, the visual function of the treated patients had been evaluated over a period of six months through two testing methods pre- and three months post-treatment and the following outcomes were noted: all three test subjects noted increased light sensitivity in the treated eye, but to different extent, and especially under dim light conditions but visual acuity was not significantly different from baseline. The study authors, who included results from two other gene therapy studies on LCA patients in their discussion, feel that the results are encouraging but need more data in order to make the therapy more efficient: identification of the retinal loci where the treatment showed a positive effect, including a determination of the type of cell contributing to the observed treatment effect (rods vs. cone photoreceptor cells), the precise measurement of the magnitude of effects across the treated area and the comparison of photoreceptor and RPE integrity at baseline and post treatment. Also, the use of sensitive noninvasive testing is encouraged in cases of treatment failures, as to better understand and prevent mistakes in future usage. The significant improvements in visual sensitivity reported 3 months after treatment were unchanged in the same patients in a 12-month follow-up as reported in a brief follow-up study, "Human RPE65 gene therapy for Leber congenital amaurosis: persistence of early visual improvements and safety at 1 year." by Cidacyian et al. [79]. The conclusion of the authors is that the safety and efficacy of human retinal gene transfer with

rAAV2-*RPE65* vector extends to at least one year post treatment [79]. These two studies addressed the need for more data on the subject of gene therapy using AAV to treat LCA, considering that the two studies before, which the authors make reference to, Maguire et al. (2008) [77] and Bainbridge et al. (2008) [78], , did not come to a unified conclusion. Thus, they are studies of high relevance, even though there were only three patients enrolled. Nonetheless, it remains to be seen how long the treated retinal cells will survive in older patients, considering that the tissue is already degenerated. Also, a correlation between dose and response has yet to be established. The authors note that in order to make an estimation of the response, the photoreceptor loss, details of injection site, vector dose and volume and genotype must be analyzed because all these factors contribute to the success or failure of the therapy [79]. Later phases of clinical trials on this topic should provide more data and answers.

Currently there is an ongoing Phase III clinical trial sponsored by Spark Therapeutics, ClinicalTrials.gov Identifier: NCT00999609, where the safety and efficacy of subretinal RPE65-gene transfer by an AAV2 vector is being investigated in 24 subjects. The study started in 2009 and has yet to publish results [64], but Spark's chief scientific officer Kathy High says that her team already has proof of long-lasting therapeutic effect up to 8 years post-treatment [80]. As stressed in the chapter "Regulatory aspects" of this paper, one can notice that authorities are still wary about this novel therapeutic approach and reticent in issuing marketing authorizations, but considering the rarity and gravity of the disease and at the same time the special immunological situation of the eye, receiving a marketing authorization for this gene therapy is plausible, if the collected phase III data is good, so Leber Congenital Amaurosis and Gene Therapy might make headlines soon.

4.2.2 Hemophilia

Hemophilia is an X-chromosome-linked bleeding disorder caused by mutations of coagulation factor VIII (hemophilia A) or IX (hemophilia B). Hemophilia A is the more common form, though both diseases have similar clinical manifestations. Severe disease is defined usually as a plasmatic level of the clotting factor below 1% of normal, which leads to spontaneous bleeds into joints and other soft tissues. Factor levels of 1-5% correlate to a milder form of hemophilia, with far

less episodes of spontaneous bleeds [81]. Since internal bleeds, like intracranial bleeds, can lead to death and repeated bleeding into joints to joint damage, the standard therapy is now prophylactic (though some patients receive only critical, on-demand administration). Patients are administered a plasma-derived or recombinant clotting factor VIII or IX. The plasma-derived option was used through the 1950s and '60s, at first in the form of fresh frozen plasma (FFP), which required infusions of big amounts of plasma in order to stop any bleeds [82]. Then the cryoprecipitation technology emerged, which enabled the creation of a clotting concentrate. Subsequently, scientists discovered a method to separate factor VIII and factor IX from pooled plasma through lyophilization. This led to the option of home treatment in the 1970s, but through the pooled plasma from thousands of donors hepatitis, HIV and other viruses got spread though the hemophiliac population [82]. Advances have been made since then in improvements in plasma control and plasma products, the safety of which has since then also improved by increased rigorous plasma testing of donors and plasma pools, as well as by introducing viral inactivation/reduction steps in product manufacturing [83]. Also, recombinant therapy emerged. Nonetheless, the treatment implies a 2-3x weekly infusion of recombinant factor VIII/IX in patients, with associated treatment costs of around 250.000$/year [57]. Being a monogenic disorder [81], hemophilia became a possible candidate for a success story in gene therapies. Endogenous FIX production would improve patient compliance in comparison to standard prophylactic therapy and shows no FIX inhibitors formation in the clinic, as have concluded some studies mentioned in this chapter [32]. An improvement in patients' health is noted starting with as low as 1% of normal FIX activity, thrombotic risk being absent in mouse-models even in a five-fold higher FIX concentration [84], thus displaying a broad therapeutic margin and giving scientists a comfortable area to experiment with this new therapy. Studies in animal models, like mice or dogs, using viral vectors for the introduction of a FVIII/FIX transgene showed sustained levels of clotting factors and a lack of spontaneous bleeds [52] sparking a scientific interest on the applicability in humans. Nonetheless, though early clinical studies determined the safety of the therapy, FIX was expressed only transiently, because of the immune clearance of transduced cells [32].

Despite the limited packing capacity of AAV, which is much smaller than, e.g., that of an adenovirus, scientists have figured out ways of modifying transgenes and associated regulatory

sequences so that they are smaller and fit to be packed into AAV capsids. For example, the cDNA coding for the Factor VIII cannot be incorporated into an AAV capsid. Nonetheless, the B domain-deleted (BDD) FVIII can be expressed and is a functional protein, used also in recombinant therapy [70]. The work on murine and canine species highlighted the use of alternative serotypes for tissue specific treatment [52]. While factors VIII and IX are normally synthesized by hepatocytes, muscle fibers are also capable of producing these proteins in their biologically active form [70]. Long-term transgene expression 2-10 months after injection of an AAV2 vector carrying the FIX gene into skeletal muscles was demonstrated in a dose escalation phase I study in year 2000 [85]. A good safety profile of this therapeutic approach has been demonstrated, in that there was no evidence of vector sequence in the patients' semen and none of the subjects developed antibodies to FIX. Nonetheless, plasmatic FIX levels were below 1% and a further dose escalation would imply a very large number of injection sites [85].

In like manner, Catherine Manno set out on researching AAV-mediated gene transfer into skeletal muscles and subsequently in the liver of hemophilia patients. Further, two studies will be discussed. "AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B", published 2003 in Blood by Manno et al., [86] is a study which talks about toxicity and efficacy of gene therapy for severe hemophilia B tested on eight patients. The background for this study are results from non-clinical studies, confirming that intramuscular injections with factor IX-carrying AAVs lead to long-term transgene expression in mice and dogs [87] and no previous clinical studies regarding parenteral injection of rAAV. Criteria for study participants were levels of FIX under 1% as a result of a missense mutation in the gene of interest. Being a dose-escalation study, patients got enrolled only after the patient dosed before them was observed for a period of at least 2 weeks. The lower- and medium-dosing cohorts consisted of 3 patients each and two patients were enrolled in the highest dosing cohort, patients were recruited from North and South America. Infection with HIV or hepatitis C did not exclude subjects from being admitted to the study. The administered dose was set at 1.5×10^{12} vg/site, intramuscularly, first cohort receiving 20-30 injection sites in skeletal muscles, a total of 2.0×10^{11} vg/kg, the high dose cohort receiving up to 90 injections of the investigational medicinal product, summing up to a dose of 1.8×10^{12} vg/kg. The main outcome was that a vector dose of 1.8×10^{12} vg/kg intramuscularly was well tolerated in all subjects. Second outcome, that there were no

vector sequences detected in the patients' semen, indicating that germ-line transmission is not a concern with AAV2. Also, none of the patients developed antibodies to FIX, muscle biopsies confirming the gene transfer and expression of the FIX transgene. Results were positive for FIX expression for up to 10 months in 8/10 biopsies. Examining muscle samples with immunofluorescent method showed a mosaic-pattern in FIX distribution, with transduced cells next to tissue blocks negative for transgene expression. Nonetheless, levels of FIX remained under 1% 14 days after FIX concentrate administration in all-but-one subjects treated, though four subjects had higher values than their baselines at 4-8 weeks after treatment. Subject A and subject B both reported a decrease of 50% in factor concentrate usage two years after gene transfer. One other observation made by the authors of this study is that even people with pre-existing neutralizing antibodies to wild-type AAV had evidence for gene transfer and/or expression, suggesting that transduction is not blocked because of these antibodies. The correlation between dose and effect could not be clearly established, arguably because of the low dose administered to the test subjects. And because of the low efficacy, with only two out of eight patients showing a slight elevation of FIX levels (more than 1% but still under 2%), the study was not considered a complete success in treating hemophilia, however findings from this study might impact research for treatments for other diseases, like muscular dystrophies, where lower levels of protein produced in skeletal muscle cells might suffice for an outstanding improvement in the patient's condition [86]. In this study, researchers have tried to administer rAAV parenterally in humans for the first time so study outcomes are interesting regardless of the clinical significance of the results. One could argue that the results might be biased because of the open-label study design but I don't think it's the case, seeing that the effectivity of the therapy was measured in gene transfer (measured by Southern blot) and transgene expression (measured by immunohistochemical staining), both objective parameters. Anyways, limitations imposed by the necessary increase in dose volume would make later phase clinical trials with this approach unlikely. So Manno and her team changed strategies and tried out a different approach.

The follow-up study by Manno and her team, entitled "Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response." [32] published in Nature in 2006, reports results from a dose-escalation trial in humans of a therapy using AAV2 FIX injected into the hepatic artery of participants. Background for this study were

very promising pre-clinical results using this method to cure hemophilic dogs with a single vector infusion into the portal vein [88], where long lasting effects were observed. The study was performed on men suffering from severe hemophilia B, including those who previously suffered of a hepatitis C infection, split up into three dosing cohorts. Lowest cohort dose was 8.0×10^{10}, middle dose of 4.0×10^{11} and the highest dose group receiving 2.0×10^{12}vg/kg. The main outcome is that doses of up to 2.0×10^{12} vg/kg do not have acute or chronic toxicity, therapeutic levels of FIX can be achieved through this method but usually drop after 8 weeks. The following results are presented in the study: Subject A died of a natural cause not related to the treatment; except for this, the only adverse event experienced by two participants was an elevation in liver enzymes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) beginning 4 weeks post-infusion, indicating a liver inflammation. Subject E, of the highest dosing cohort, showed FIX levels of 11.8% two weeks after vector infusion, his fellow high-dose cohort patient showed 3% levels of FIX, which returned to the baseline 4 weeks post-infusion. Other results were under therapeutic levels, indicating a minimal effective dose of 2.0×10^{12}vg/kg in humans. Decrease in FIX levels coincided with the increase in transaminase levels in some patients, indicating an immune-mediated elimination of transduced hepatocytes. Another factor which was identified as relevant in dose-responses is the pre-existing neutralizing antibodies (NAB) titer[4] to AAV, because both patients from the high dose cohort received the same dose and had, however, different FIX levels. Patient E had a pre-treatment antibody titer of 1:2, while patient F had 1:17, the difference being obvious in the degree of expression of FIX (11% vs 3%). Though a high elevation of AAV-neutralizing antibody titer was noted in all subjects, none of them developed antibodies to FIX. The research team concludes that the immune response responsible for the clearance of the transduced cells could be reduced with a short-term immunomodulatory pharmaceutical regimen, finally allowing a long-term transgene expression. This is merely a postulation from the team, not based on data but on observations about the cascade of immune response to capsid antigens in humans: peptide sequences of the capsid were identified as having a high binding affinity to the major

[4] The titer is the concentration of an antibody, as determined by finding the highest dilution at which it is still able to cause agglutination of the antigen.

histocompatibility complex class I (MHC-I) molecule. These molecules, in turn, show great affinity to $CD8^+$ T-cells. If the T-cell (also known as killer T-cell) recognizes the antigen presented by MHC-I on the transduced cell's membrane (because, for example, the person was previously infected with AAV) it kills the cell [89]. The risk of this happening is high, since most humans are naturally infected with AAV and helper virus in childhood [32]. The authors came to the conclusion that this chain of events could hypothetically be halted with the use of immunosuppressive drugs until the viral capsid particles are naturally eliminated from capsid-harboring cells. This would mean the preservation of infected cells and, in turn, the long-term expression of the transgene of interest. What we can learn from this study is that health care professionals must lookout for immunological responses to AAV both for patient safety but also for the increase of the therapy's efficacy. Manno and her team once again got very close to a clinical breakthrough and the findings regarding immunological responses, previously not reported in animal models, provide very important new information for this field of research. The study design itself is similar to the previous study [86], around the same number of participants, all with severe hemophilia B, split up into similar dosing cohorts. Yet the main difference is, that the administration route is not intramuscular anymore and the target tissue is the liver, as opposed to skeletal muscle in the 2003 study. The one serious drawback is the lack of long-term effect, which is desired in a gene therapy.

Information gathered from previous studies on the sustained high expression of FIX after injection of AAV into the portal vein or hepatic artery into animals and humans and the emergence of self-complementary AAV (scAAV[5]) prompted a phase I/II clinical trial using scAAV8FIX by Amit Nathwani et al., the "Adenovirus-associated virus vector–mediated gene transfer in hemophilia B." study from 2011 [90]. The central question that was addressed by the authors is whether or not gene transfer is a suitable option for patients suffering from severe hemophilia B. The study background, as mentioned by the authors, are many points previously mentioned in the introduction to this sub-chapter.

Six patients suffering of severe hemophilia B and lacking neutralizing antibodies to AAV8 were recruited for the study, patients 1-5 recruited in 2010 and patient 6 in 2011. The study was

[5] It is theorized that scAAVs skip the second strand synthesis required in order for genes to be expressed, by delivering a duplex genome whilst improving transduction efficiency.

performed at the Royal Free Hospital in the UK, where the study participants were split into three dosing groups. The low-dose group received a dose of 2 x 10^{11} vector genomes (vg)/kg, the intermediate group 6 x 10^{11} vg/kg and the high dose group 2 x 10^{12} vg/kg. The team used a codon-optimized FIX (FIXco) expression cassette that is packaged as complementary dimers within a single virion (scAAV). The scAAV vectors mediate transgene expression at higher levels than do single-stranded vectors [91]. The vector used in this study was also pseudotyped[6] with a capsid of serotype 8, which shows a lower seroprevalence in the human population than the serotype 2, thus lowering its immunogenicity. One more advantage of the AAV8 is its tropism for the liver, making a single intravenous injection the preferred way of administration, which is safer for the hemophiliac patients in comparison to multi intramuscular injections.

The vector was persistent in the subjects' bodily fluids and analyzed tissues up to 15 days post gene transfer with no change in the wellbeing of the patients. There were a total of three adverse events, two participants developed anemia at 5-7 weeks post gene transfer and another test subject suffered from a transient period of bradycardia. The results of this phase I/II clinical trial are promising: 4/6 patients discontinued FIX infusions without experiencing severe hemorrhage, even when they undertook activities that had provoked bleeding in the past. The other 2/6 had much longer intervals between injections and it was noted that both had severe hemophilic arthropathy. 0/6 patients had an immunologic response to the FIX transgene product and the highest capsid specific T-cell count was reported in patient 4 at 4 weeks after gene transfer. Results suggest that the magnitude of immunological response to AAV particles depend on vector dose, individual variations in vector processing and presenting, and the patient's exposure to wild-type AAV. The authors conclude that routine glucocorticoid prophylaxis is not indicated, since incidence and timing are uncertain and early intervention has proven to be effective.

The main outcome was that a single, peripheral vein infusion of scAAV2/8-LP1-hFIXco consistently led to long-term expression of FIX transgene at therapeutic levels, without acute or long-lasting toxicity. The patients in the high dose cohort showed the highest levels of factor expression, in patient 5 7% of normal values were maintained seven weeks without additional

[6] Pseudotyping is the process of producing viruses or viral vectors in combination with foreign viral envelope proteins. With this method, the foreign viral envelope proteins can be used to alter host tropism or an increased/decreased stability of the virus particles.

prophylactic therapy and patient 6 showed 8-12% of normal levels up to eight weeks post infusion. Further outcomes include that immune mediated, AAV-capsid induced elevations in alanine aminotransferase (ALT) levels remain a concern but data suggests that this can be controlled by a short course of glucocorticoids without loss of transgene expression.

There was still evident lack of long-term data on this novel therapeutic approach, so the study "Long-term safety and efficacy of factor IX gene therapy in hemophilia B." , published in 2014 by Nathwani et al. [57], investigates the long-term effects of the scAAV2/8-LP1-hFIXco therapy in the six patients enrolled in the 2011 study and four additional patients. The central question is whether or not this therapy shows long-term beneficial effects, the vector-dose relationship and the level of persistent or late toxicity.

The participants were followed-up up to 3.2 years after vector administration through extensive clinical and laboratory monitoring. The four new patients, also suffering from severe hemophilia B, were enrolled in 2012 and each received a high dose of 2×10^{12} vg/kg of vector, administered intravenously in a peripheral vein. After administration, the patients were monitored in terms of plasma factor IX activity, vector shedding and humoral/cellular immune responses against the capsid but also against the transgene product. Additional routine laboratory studies were performed. The primary outcome was that the vector infusion at all three doses is safe to use in humans. The efficacy (secondary outcome) was assessed by three parameters: factor IX activity, the annual number of bleeding episodes, determined also prior to study for each participant, and the annual amount of FIX concentrate being administered. All of the 10 participants had 1 to 6% of normal FIX plasma activity for up to 3.2 years post infusion. This effect seems to be vector dose-dependent, with patients in the highest dose cohort showing a steady 5.1^{+}-1.7% FIX activity, compared to 1.8+-0.7% in the lowest dose cohort. The annual amount of factor IX concentrate that was administered to the patients dropped by 96%. Despite the much lower factor use, all patients showed a decrease in bleeding episodes in the year post-gene therapy of relative 90%. The highest dosing cohort experienced a decrease in bleeding episodes of 94%.

There have been 18 adverse events reported which are related to the study agent, the most common being an increase of the alanine aminotransferase (ALT) enzyme in the liver, which occurred in 4/6 patients of the high dose group. This increase in serum ALT has been correlated

with a decrease in factor IX levels, indicating a loss of transduced hepatocytes and overall diminishing the therapy's efficacy by reducing FIX levels 50-70%. The ALT level was reduced back to normal by a short prednisolone course. The authors do not justify their choosing of prednisolone as an immunosuppressing agent over other therapeutic options, though Prednisolone "[It] is drug of choice for systemic anti-inflammatory and immunosuppressive effects" [92]. The study successfully identified weeks 7 to 10 post gene transfer as the critical period of monitoring and pharmacological intervention and argues that an improvement in the potency and quality of the vector would lower or even eliminate its liver toxicity.

The vector genome was detected in saliva, urine, semen, stool or plasma for up to 6 weeks post gene transfer, though none of the patients reported symptoms associated with viral infections, like fever or sweating. This is in-line with pre-clinical data, where AAV-particles have been detected in secreta and excreta of macaques for up to one and a half months post-treatment [93]. Vector shedding data is crucial in clinical trials so as to assess whether the product poses a risk of transmission of viruses to untreated individuals [94]. It is also a part of the environmental risk assessment of the product's marketing authorization application [94]. The clinicians also reported increased anti-AAV8 IgG antibody levels in treated patients for at least 12 months post-infusion; an asymptomatic parameter relevant only in the case of another course of AAV8 based treatment. They also note, that during the study the patients got 3 million units less FIX concentrate, resulting in financial savings of more than $2.5 million based on 2014 prices. This result is relative, considering that the investigational medicinal product does not yet have a price tag, so a price comparison assessment is not yet possible. Concluding, the authors feel that the results of this study are encouraging: the administration of this gene therapy in humans brings advantages to the patients suffering from severe hemophilia B (durable FIX expression and improvement in the bleeding phenotype) while showing few, non-critical vector-related adverse effects. These two studies symbolize a milestone in gene therapy, presenting statistically and clinically significant results after systemic AAV mediated gene transfer into humans whilst also demonstrating the safety of this therapeutic approach. The probability of coming to an erroneous conclusion because of chance, bias or confounding are very slim, because this study, much like the studies conducted by Manno [86; 32]and presented beforehand, uses objective

criteria to assess the therapy: circulating FIX levels, vital signs, anti-capsid and anti-FIX antibody levels, cellular immune response and vector shedding.

Currently, companies are competing to get a gene therapy targeting hemophilia on the market. Baxalta and UniQure have already produced early clinical data in hemophilia B by 2016's first quarter, followed by Spark therapeutics and Dimension Therapeutics, which also have products in development. But in the search for a cure for hemophilia A, BioMarin is the first and only company to have reached clinical testing for their product. The company edited the gene coding for factor VIII, which is much bigger than the gene coding for the Factor IX, to be shorter and so to fit into the adeno-associated viral capsid used as a vector [95]. The results as of 20.04.2016 are astonishing: from the 8 patients enrolled, the 6 patients dosed in the highest dose cohort (6 x 10^{13} vg/kg) expressed therapeutic levels of FVIII, ranging between 4% and 60% of a healthy individual's plasmatic level [96]. Patients in the lower dosing cohort didn't achieve therapeutic levels above 1%, even though the lowest starting dose (6 x 10^{12} vg/kg) was higher than the one demonstrated by Nathwani [57] to achieve therapeutic levels in hemophilia B patients (2 x 10^{12} vg/kg). The observations are ongoing and the high-dose cohorts have been monitored only for 5-16 weeks, so long-term data is still to come. As expected, an elevation in liver enzyme levels occurred in the patients who did not receive prophylactic corticosteroid treatment, prompting the investigators to administer prophylactic treatment to all patients dosed after patient 3, who suffered of a mild increase in ALT levels at week 4 post-treatment. After this modification to the study protocol, no abnormal ALT levels were reported in any of the subjects [96]. BioMarin declared, that plans for Phase III clinical trials and for high volume manufacturing are underway [96], in the hope that further clinical data will be favorable and their medicinal product will be granted a marketing authorization. If so, hemophilia B patients might finally profit from over 15 years of gene therapy research for hemophilia and benefit from what this innovative therapy, based most frequently on AAV, has to offer.

4.3 Serious Adverse Events

Since its first clinical trial, AAV has been administered as a vector carrying a gene of interest to more than 600 people worldwide [97]. According to the Recombinant DNA Advisory Committee

(RAC), by 2007 only a total of 29 adverse events have been reported from AAVV clinical trials in the US, none of which serious [97]. By 2015 no serious adverse events had been reported as a result to AAV gene therapy [58]. A case which did raise awareness was the death of a female patient enrolled in a 2007 gene therapy clinical trial using AAV2 against rheumatoid arthritis. Rheumatoid arthritis is an autoimmune condition, which causes inflammation in the joints. The patient had been administered the investigational medicinal product and died some weeks after, being the first human death reported in an AAV clinical trial [98]. The FDA put the trial on hold until more information was gathered and postmortem findings indicated a massive fungal infection with *Histoplasma capsulatum*, thus deeming the patient's death as unrelated to the gene therapy and allowing the trial to move forward, with only little changes to the clinical trial protocol [97]. Not only is AAV already being tested in clinical trials for beforehand mentioned conditions, therapies for other disorders using this vector are in pre-clinical phases and show some potential. Researchers' interest in AAV applicability spreads from lysosomal storage disorders to muscular dystrophy, from neurological diseases to the therapy of cancer. So one thing is clear about the future of AAV vectors in gene therapy: much is yet to come. Of course, regulating authorities impose some limitations in AAV research, as discussed in the next chapter, "Regulatory aspects".

TABLE 2: *REGISTERS OF GT CLINICAL TRIALS, USEFUL LINKS*

US Government Database	http://clinicaltrials.gov/
NIH Clinical Center	http://clinicalstudies.info.nih.gov/
EU Clinical Trials Register	https://www.clinicaltrialsregister.eu/
Wiley Gene Therapy CT Worldwide	http://www.abedia.com/wiley/index.html
International Clinical Trials Registry Platform (ICTRP)	http://www.who.int/ictrp/en/

5. Regulatory aspects

5.1. General

Drugs and medical devices for human use are among the most strictly regulated products in the developed world. This has happened gradually over longer periods of time, usually as a response to a catastrophe caused by use of drugs with very little labelling information on potential risks and side effects. Thus, the fundamental purpose of regulation is the protection of public health [99].

Gene-therapy medicines are categorized in Europe as Advanced-therapy Medicinal Products (ATMPs), a category which also includes somatic-cell therapy medicines, tissue-engineered medicines and combined ATMPs (these contain one or more medical devices as an integral part of the medicine) [100]. As for any new technology, the regulatory authorities adopt a flexible approach toward the control and research of these products in their guidelines, so that any step during research, development and post-marketing monitoring activities can be easily modified in light of new information gained through experience in production and use [101].

5.2. Marketing Authorization Application

There exist guidelines on gene therapy products for example by the European Medicines Agency (EMA) [Table 3], which regulates medicinal products in Europe, or by the United-States based Food and Drug Administration (FDA), which regulates not only medicinal products but also food safety, tobacco products and cosmetics in the United States (http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/) [102]. These are bodies of work regarding special considerations on ATMPs meant as recommendations and not as fixed laws. The agencies try to stimulate the expansion of viable scientific information on this subject not only through these guidelines, but also through scientific advice meetings, upon request from the researching party. In Europe, special pricing, with up to 90% off the price of the meeting, is offered to non-for-profit organizations and small and medium sized enterprises (SMEs) so as to make development

of ATMPs easier and more feasible for clinical trial applications and marketing authorization applications [103]. The Agencies help researchers plan appropriate testing and studies in the non-clinical and clinical phase by answering companies' questions with the ultimate goal of delivering safe and efficacious therapies to patients in need. Scientific advice can be sought at any given point during research & development, at national or multi-national agencies. Scientists should choose the agency with the most knowledge and experience in their specific area of work so as to ensure the most comprising input [104].

If looking to develop and market a gene therapy in the USA, it is advisable to seek an open dialogue through scientific advice with the Food and Drug Administration (FDA), since they authorized only one gene transfer product (the talimogene laherparepvec IMLYGIC, Amgen Inc.) in October 2015 [105]. In spite of this milestone, Uniqure, the manufacturer of Glybera (alipogene tiparvovec), decided to stop pursuing a marketing authorization for Glybera in the US in December 2015. The reasons invoked were FDA's high demands for the MAA, like requirements for more data from further clinical trials on the LPLD therapy. These demands were issued regardless of the medicine already being approved in Europe since 2012 [106].

Some national agencies in Europe might employ experts in the field of gene therapies or even scientists who specialize their research on AAV vectors. It is advisable to do research on the members of the commissions before seeking out scientific advice, as to pin-point an agency which is most likely to offer valuable input. The German Paul-Ehrlich-Institute (PEI) is an agency of the German Federal Ministry of Health, specializing in biological medicinal products (www.pei.de). Also, the MHRA, Medicines & Healthcare products Regulatory Agency, is an executive agency of the Department of Health, which provides scientific advice on advanced therapies and is regarded highly amongst national agencies (https://www.gov.uk/government/organisations/medicines-and-healthcare-products-regulatory-agency). If a company or developer seeks to obtain a more aligned advice across Europe in developing the drug for the – mandatory – centralized marketing authorization procedure – he should seek centralized scientific advice from the Scientific Advice Working Party (SAWP)/CHMP at EMA[107].

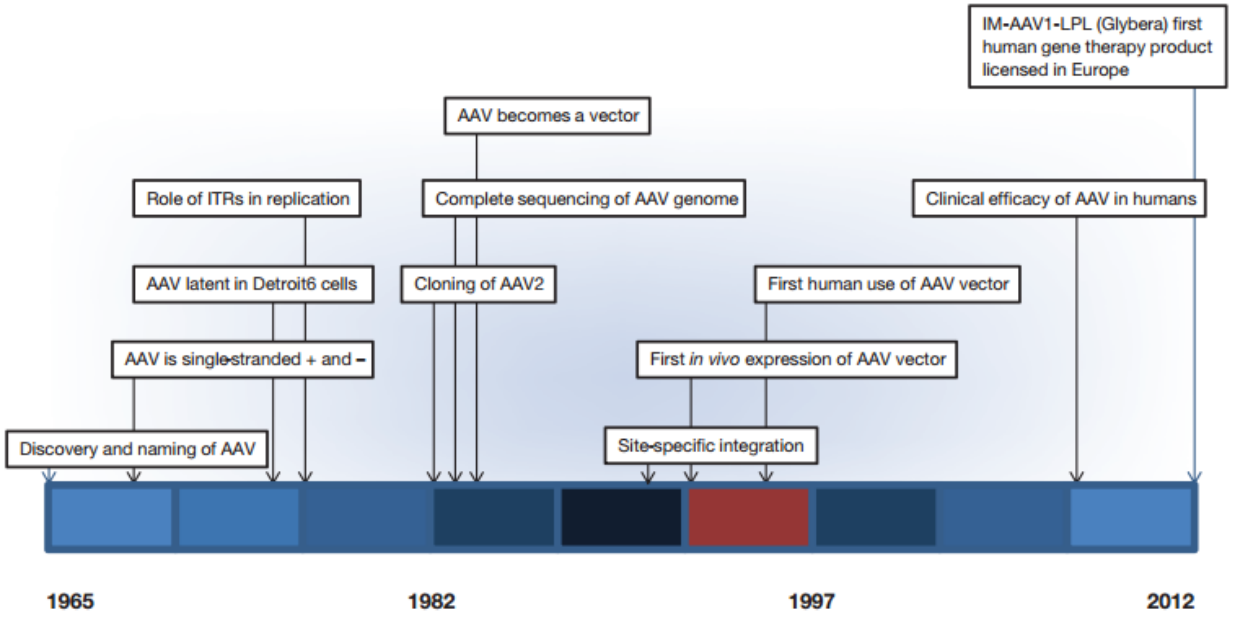

FIGURE 7: MILESTONES IN AAV BIOLOGY AND SUBSEQUENT VECTOROLOGY, FROM THE VIRUS' DISCOVERY TO ITS BREAKTHROUGH ON THE MARKET 47 YEARS LATER. IM, INTRAMUSCULAR; ITR, INVERTED TERMINAL REPEAT (SOURCE: FLOTTE, TERENCE R. "BIRTH OF A NEW THERAPEUTIC PLATFORM: 47 YEARS OF ADENO-ASSOCIATED VIRUS BIOLOGY FROM VIRUS DISCOVERY TO LICENSED GENE THERAPY." [3]).

5.2.1. Europe

The European Commission publishes laws, the pharmaceutical legislation being available to the public online, in a body of work called EudraLex, organized in 5 volumes regulating the medicinal products in the European Union (http://ec.europa.eu/health/documents/eudralex/index_en.htm). In volume one, sub-section 3.2., the specific requirements for gene therapy medicinal products are briefly addressed. Regulation EC 1394/2007 [108] on advanced therapy medicinal products acknowledges therefore the great potential these novel biomedicines possess in treating diseases and disorders of the body. This regulation adds provisions to those in Directive 2001/83/EC and announces the creation of a Committee for Advanced Therapies (CAT) in order to provide sufficient expertise on this multi-disciplinary field [108]. ATMPs have to follow a centralized procedure route when seeking a marketing authorization, which means, that the developers need to submit their documents to EMA only once, and the European Commission will grant a MA valid throughout the European Union or won't grant it, which means the product is not allowed anywhere on the

EU market [108]. The Commission will come to a decision based on the CAT's input and expertise and the CHMP opinion [108]. The CAT also provides input on whether a product falls into the category of "advanced therapy medicinal product" or not, since this can be a grey area for e.g. medical devices [109].

The beforehand mentioned Directive 2001/83/EC [100] details deeper special requirements for gene therapy medicinal products in the area of the *finished product, active substance, pharmacology, pharmacokinetics and toxicology thereof,* and many more general considerations on gene therapy using viral vectors, but not going into specifics for the AAV.

TABLE 3: SPECIFIC GUIDELINES ON GENE THERAPY ON EMA'S WEBSITE (SOURCE: *WWW.EMA.EUROPA.EU* [110]).

• The **overarching guideline** for human gene therapy medicinal products is the Note for guidance on the quality, non-clinical and clinical aspects of gene transfer medicinal products (CHMP/GTWP/671639/2008)
• **Questions and answers** on gene therapy (EMA/CHMP/GTWP/212377/08)
• Guideline on scientific requirements for the **environmental risk assessment** of gene therapy medicinal products (CHMP/GTWP/125491/06)
• Reflection paper on **design modifications** of gene therapy medicinal products **during development**(EMA/CAT/GTWP/44236/2009)
• Reflection paper on quality, non-clinical and clinical issues relating specifically to recombinat adeno-associated viral **vectors** (CHMP/GTWP/587488/07)
• ICH Considerations - **Oncolytic Viruses**(EMEA/CHMP/ICH/607698/2008)
• Guideline on quality, non-clinical and clinical aspects of medicinal products containing **genetically modified cells**(CAT/CHMP/GTWP/671639/2008)
• Guideline on the non-clinical studies required **before first clinical use** of gene therapy medicinal products (EMEA/CHMP/GTWP/125459/2006)
• Guideline on non-clinical testing for **inadvertent germline transmission** of the gene transfer vectors (EMEA/273974/2005) • Reflection paper on management of clinical risks deriving from **insertional mutagenesis** (CAT/190186/2012)
• Guideline on **follow-up of patients** administered with gene therapy medicinal products (EMEA/CHMP/GTWP/60436/2007)

The EMA published different guidelines on gene therapy [**Error! Reference source not found.**] but a paper which does address AAV vectors directly is **EMA's "Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral**

vectors" [10]. I will present the main points shortly, as to better exemplify the general tone of this paper and its contained information.

The European Medicines Agency created this guideline for the safe research and use of AAVVs. After addressing some manufacturing methodologies used to produce rAAVs, the paper touches important **quality considerations**. It stresses the fact that all cell lines used in the manufacturing process should follow a cell bank system and thus fall under the European pharmacopoeia monograph 5.2.3. principles, which are mandatory to consider in control processes. The tests should particularly address contamination by the *wt*AAV, but also any viruses identified as helper virus for AAV replication, taking into consideration the risk of possible replication of these viruses in the cell line used for AAV manufacture. The helper viruses used should themselves be produced from a seed lot system using a qualified cell line and information about their origin and further manipulation should be documented. Special attention should be given to the content of replication competent viruses when using recombinant helper viruses, as these might be generated during production steps of AAV medicinal products.

Wild-type AAVs and novel replication competent viruses should be regarded as impurities, when designing non-clinical and clinical trials, as these can impact the safety of the product.

The discussion continues with specific measures to be taken based on the different production systems. In **virus containing production systems** there is an encouragement to characterize and qualify for use any helper viruses before they get used in the AAV production processes. The European Pharmacopoeia (Ph. Eu.) general chapter 5.14 Gene Transfer Medicinal Product for Human Use is a good starting point for guidance regarding testing programs/quality specification selection for the quality of the helper virus/viruses [26]. These helper viruses must be inactivated through a method shown to be effective, yet even so the DNA contained by them might contaminate the final bulk or drug product. An upper content limit of helper/hybrid virus should be therefore included in the final bulk or drug product specification, if the chosen virus inactivation cannot be validated. When a helper virus is used, which contains Rep sequences- required for DNA replication- it is recommended to quantify the DNA content in terms of contaminating Rep sequences, since unintentional rAAV genome replication might occur.

Virus free production systems have the advantage of not being contaminated with helper/chimeric viruses, since the transfection happens with the help of plasmids. This method has the drawback of a naturally occurring variability since the transfection process itself is subdue to an intrinsic variability. Materials used such as E. coli plasmid master cell banks, transfection reagents etcetera, should be qualified. The scale-up of this process is difficult and the transfection conditions should be analyzed and optimized at each manufacturing scale since the scale of the process has been shown to impact the amount of replication competent (rc) AAV generated. When employing a strategy using packaging cell lines, one should show the genetic stability of the construct. Also, when a method of transcriptional control is being used, the induction agent must be removed through a validated purification strategy, or a content limit should be incorporated in the release specification.

In the chapter **Quality control of the product**, the paper addresses the **Product content/titer** first. Quality control release specifications are usually a mix between two titration methods: quantitation of DNA amplification and transgene expression after transduction and co-infection with the helper virus. This touches on two important quality parameters when using an AAV vector, firstly, the biological activity measured as the transgene expression and secondly, the ability of the DNA to enter cells/nuclei. A more exact measure of full particle to inactivity ratio could be given through the combination of DNA quantification of DNase[7] resistant particles and an appropriate rAAV titration method. The **purity** of rAAV vector lots should be evaluated through assays used to detect process impurities and potential contaminants, like plasmid DNA, helper/chimeric virus DNA or cellular proteins, which can be immunogenic. Also, empty capsids and uninfectious particles can activate the immune system, thus counting as impurities, which need to be quantified in such a way, as to get the same amount of "antigenic load" when the drug is administered to patients. Encapsulated residual DNA impurities, e.g. plasmid or helper virus DNA, need to be well investigated as to evaluate their potential of protein expression. In some cases, for example when vectors contain persistent, auto-replicating episomal[8] co-

[7] *Deoxyribonuclease is an enzyme that catalyzes hydrolytic cleavage of phosphodiester linkages in the DNA backbone.*

[8] *Extra-chromosomal genetic material that may replicate autonomously or become integrated into the chromosome*

packaged DNA, a comprehensive risk assessment might be requested so as to demonstrate the long-term product safety.

The next chapter discusses **non-clinical evaluation**, starting with the **choice of animal model.** The agency recognizes the possibility of applicability of species specific vectors and/or species-specific transgenes in animal models, because the AAV is a species specific virus, which means that it is possible that the biodistribution of a human serotype derived vector looks different in an e.g. mouse-model then in humans. Also, this approach generates more useful information about immune responses in individuals previously exposed to the wild-type virus. The transgene used in the clinical trials is likely to be of human origin and thus the agency considers it justifiable to use the homologous animal gene in non-clinical settings, so as to minimize the impact of immune responses. An immune response might, for example, trigger a clearance of the expressed protein and as a result lead to incorrect data interpretation on the efficacy of the transgene product. Therefore, it is deemed appropriate to use a serotype of virus that is specific to the animal model of choice and/or species-specific transgenes in pivotal non-clinical studies, preferably after seeking advice from the regulatory authorities. Numerous animal species have been used so far in non-clinical studies, like dogs, mice, rhesus monkeys or non-human primates, however it might be necessary to use more than one species for a comprehensive non-clinical development scheme since it is uncertain which model works best.

Issues on **vector persistence** are many and available information is scarce, respectively fluctuating depending on many variables. The investigator's attention is brought to issues like **insertional mutagenesis**, which became a concern after the publishing of a study showing an increased rate of hepatocellular carcinoma in neonatal mice treated with a rAAV [55]. This study does not definitively confirm the oncogenicity of these vectors but reveals the necessity of an assessment on the extent of the vector integration in-vivo but also in vitro. Also, the mechanism of long-term gene expression should be investigated i.e. integrated or episomal, as this plays a role in the duration of vector persistence and protein expression of the "gene of interest". The level of expression of this gene should be investigated in order to correlate a decline in vector levels in both target and non-target organs with protein levels. For the **environmental risk assessment** it must be taken into account that rAAV vector particles have been detected in the Macaque for up to one and a half months in-vivo after administration [93], when designing

shedding and biodistribution studies. Studies have found that **shedding** of rAAV does occur and that vector DNA can be found in saliva, serum, urine and semen starting with the first day post-administration up until a number of weeks afterwards in serum. The infectious virus quantification should be carried out on gathered samples and should be used in the environmental risk assessment as part of the Marketing Authorization Application (MAA).

It has been shown that the **tropic behavior** of a rAAV vector serotype can vary from species to species, therefore tissue tropism data acquired in non-clinical trials should be regarded critically when translating the data to humans.

Reactivation of productive infection can occur if the subject is infected with both wild-type AAV and a helper virus. Furthermore, viral particles of serotype 1 have been demonstrated in the serum up to 3 months post i.m. injection [93], therefore indicating a great probability of an immune response. Non-clinical studies should address these two issues, if relevant, under the special consideration of parallel medication during the clinical trial, as this might impact the biodistribution and the chance of a viral reactivation, e.g. treatment with immune-suppressants.

Gem-line transmission is also an important topic in gene transfer medicinal products. The possibility of germline transmission of vector genetic material raises ethical and safety concerns [61]. This has led to the creation of the Directive 2001/20/EC, which states that no gene therapy clinical trials may be carried out which result in modifications to the subject´s germline genetic identity. rAAV DNA has been found in gonadal mouse and rat DNA [62] therefore it is recommended that germ-line transmission studies are carried out in the context of vector DNA biodistribution. In addition, individuals enrolled in AAV gene therapy clinical trials should use a barrier contraception method for at least three months, as to minimize the risk of germ-line transmission of viral DNA, since this question has not yet been fully understood/investigated in humans.

In the chapter on **clinical considerations**, the paper starts by mentioning the fact that results from animal testing cannot just simply be adopted as true in humans. Therefore, **biodistribution and shedding** studies should be included in the clinical trial protocol. The biodistribution of the vector, though slightly dependent on the route of administration, is also prone to extensive dissemination even when applied locally (e.g. intramuscularly) and requires as such diverse

samples, e.g. but not limited to: saliva, tears, urine, feces, blood/serum, which do not always reflect the route of administration. Non-clinical shedding profiles of the vectors should be taken into consideration when determining the time interval between samples.

Extrapolation of animal data on **immunogenicity** is not easy, and special consideration should be given to whether or not the subject has pre-existing antibodies to the AAV-serotype under investigation. The formation during clinical trials of neutralizing and non-neutralizing antibodies against the vector but also the transgene should be discussed. Also, the authors of this guideline suggest giving special consideration to immune responses against vector particles or transgene product , since they present antigenic properties and may therefore spark a cytotoxic T-Cell response which eliminates transduced cells and leads to a decrease in transgene expression [32]. There exists the possibility of administering an immunosuppressive treatment to the test subjects concomitantly to the gene therapy, as to minimize a possible immune response in individuals with pre-existing immunity to various AAV serotypes, and this must be carried out as a standardization of the regimen across all study sites.

This article specifies that long-term safety and efficacy of these vectors also has to be established through long-term patient observation.

An open dialogue is encouraged between companies striving for a Marketing Authorization and the regulatory officials, since both parties still have much to learn about the use of AAV vectors in gene therapies.

The Committee for Proprietary Medicinal Products (CHMP) of the EMA elucidates in its **"Note for Guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products"**, 2001, [101] general aspects to take into consideration, when developing a gene therapy medicine. In the following section I will name a few important ones, for more extensive guidance please refer to the paper online. This guideline is currently (July 2016) under review and until the approval of the revised version, the 2001 adopted guideline remains in use[111]. For a therapeutic approach using a viral vector as the gene delivery system the CHMC requires documentation on origin, history and biological characteristics of the parental virus and detailed information on the recombinant viral genome, like in which parts of the viral genome have been inserted new constructs or what new sequences have been included. If new constructs have been

included, e.g. mutations, or deletions, the CHMP desires an explanation to the rationale of said modification. An explanation is expected for the design chosen for the viral vector, including, but not limited to the location of the transgene in the vector and its tissue expression. A scientific explanation regarding the choice of viral vector and its clinical indications should be included, based on scientific data on tissue tropism, transduction efficiency, tissue specificity of replication and the viral gene sequence of the wild-type virus. To minimize the concern on a possible recombination of the viral genes so as to become competent for replication again (in the case of replication-deficient viruses), a solid documentation of the strategy used to render the virus replication-deficient is expected. Cell lines, products and intermediates should therefore be screened for replication competent viruses (RCV).

Special attention should be given to toxicological studies. These should be designed on a case-by-case approach, in the case of AAV vectors; an animal model should be picked out where the biological response to the gene product would be similar to that in humans and therefore comparable and meaningful. The proposed clinical dosing regimen (dose, duration, frequency) should be similar for the experiments on animals to the one in humans. Transgenic/immunodeficient animals are acceptable animal models, if they provide an advantage for the meaningfulness of the results.

5.2.2. The U.S.A.

The volume of information from the FDA on AAV is limited but there are several regulations and guidance addressing the broader topic of gene therapy. In the following section, some of these will be shortly presented.

In 1998, the U.S. Department of Health and Human Services of the Food and Drug Administration (FDA) Center for Biologics Evaluation and Research (CBER) published on the governmental website its "**Guidance for Industry; Guidance for Human Somatic Cell Therapy and Gene Therapy"** [112] thus addressing an emerging topic which is becoming difficult to ignore and requires some guidance from the authorities: somatic gene therapy. This document is still relevant and revocable on the FDA website and contains general considerations on concerns for production, quality control testing, and administration of recombinant vectors for gene therapy;

and of preclinical testing of both cellular therapies and vectors. While AAVs are not explicitly addressed, concerns regarding safety of vector administration *in vivo* are raised and discussed.

The **route of administration** is relevant to vector toxicity *in vivo*. It is therefore suggested that pre-clinical data in animal models use homologous routes of administration as proposed in the clinical studies protocol. The choice of **species of animal** for the preclinical studies should be taken based on two considerations: firstly, the biological activity of the vector construct should be similar to that in humans and secondly, the species should be sensitive to infection by the wild-type virus, so as to better predict its pathogenicity in humans. The starting dose in clinical trials should be pondered based on *in vitro* and *in vivo* data gathered in the preclinical phase, with adequate safety margins. Treated animals should be monitored in order to gather toxicological data through observation on general health, serum biochemistry, hematology and pathological changes in histology. A recommendation is made to track the biodistribution of the vector through localization studies using the currently most sensitive analytical method and should include an evaluation on the gene persistence. **Germ-line transmission** risk should be assessed through pathological examination of tissue samples from animal tests and ovaries for incorporation of vector into germ cells. Lastly, the guidance addresses the need of inclusion of the patient's immune status in the risk-benefit analysis, since immunodeficient patients may be subject to greater safety risks.

The "FDA Guidance for Industry: Gene Therapy Clinical Trials - Observing Subjects for Delayed Adverse Events" addresses pharmacovigilance aspects of gene therapies, including those based on AAV vectors, stressing the fact, that even though the virus itself doesn't have the propensity to integrate or reactivate following latency and thus has a low risk of delayed adverse events, the risk of autoimmune phenomena should be considered if the vector is shown to have persistent transgene expression [113].

The scientific community is encouraged to share information with the FDA and -with the rise in interest on AAV- the probability that the agency will consider setting in place more specific guidelines and regulations is growing.

5.3. Special statuses

Scientific advances in the safety and efficacy of advanced medicinal products are slowly changing regulators' attitude towards gene therapies. As an example, in April 2014 the FDA took the decision to grant **breakthrough status** to Mydicar, a product of Celladon currently being tested in humans with the indication for patients with systolic malfunctions leading to heart failure because of a deficiency of the enzyme SERCA2a. An AAV vector is used to insert the correct gene copy for SERCA2 into the cardiac muscle cells. A study on 39 patients showed a significant reduction of 80% in heart failure when compared to placebo. A high dose of Mydicar not only shows such benefit but it was sustained for 3 years after administration of treatment [114]. Breakthrough status is granted to medicine targeting life-threatening diseases that show significant improvement over the standard of care, thus potentially fast-tracking its authorization for marketing and allowing a better communication with the FDA [115]. Indirectly, the FDA is stating the trust in this medicine and its curative potential, which is a step forward for AAV utilization.

A similar initiative exists also in Europe, called the PRIME scheme, aiming to expedite the approval process for medicines that benefit patients with unmet conditions, based on promising early clinical data[116].

There was an Adaptive Licensing pilot project, which started in 2014 and recently (as of August 2016) published a summary and key learnings on EMA's website [117]. Adaptive pathway brings medicine for unmet conditions to patients in a progressive way, meaning that the license is issued firstly for a small group of patients and gradually extends the previously authorized indication(s) based on evidence gathered over time. Two ATMPs have also entered the pilot, indicating that it is a possible option for gene therapies too [117].

Through programs like PRIME, if eligible, gene therapy developers can get early and augmented support from EMA in order to generate timely data on the medicine's benefits and hazards, while the agency takes advantage of the improved communication throughout the development process, e.g. through scientific advice, to be up-to-date with the newest developments and thus

is likely to expedite its MA decision making. The ameliorated and early communication can be especially beneficial for researching parties that have limited knowledge of the regulatory framework in Europe. [118]. A form of recognition of special status of a medicine is the so-called **"orphan drug designation"**. This is granted to medicine addressing the need of patients suffering from a rare disease and the developers get support in advancing the development and evaluation of such products. The FDA defines a rare condition as a disease/disorder which affects fewer than 200.000 people in the U.S., or that affects more people but is not expected to recover the costs associated with research, development or marketing through sales [115]. There is a program set in place, the Orphan products grant program, which provides funding for clinical research of drugs, biologics, medical devices and medical foods in rare diseases and conditions [119]. The EMA has a tighter definition, of what constitutes an orphan drug. The prevalence of the condition must not be greater than 5 in 10.000 people in the EU, must be a life-threatening or chronically debilitating condition and the medicine must provide a significant benefit to those affected by the condition, if other methods of prevention, diagnosis or treatment are available [120]. Many gene therapies based on AAV vectors have already been granted this designation [121] because they meet all requirements. To name a couple of recent designations, BioMarin Pharmaceutical Inc. has received orphan drug designation from both the FDA and the EMA for first AAV-factor VIII Gene therapy for patients suffering from hemophilia A in March 2016 [122; 123]and Applied Genetic Technologies Corporation (AGTC) also received orphan drug designation for its gene therapy AAV-based product candidate for the treatment of achromatopsia, a rare eye disease caused by mutations in the CNGA3 gene, in October 2015 in Europe [124] and November 2015 in the United States [125].

It would appear that AAV use in emerging gene therapies has made its way on the radar as being a possibly valuable therapeutic option, sparking a wave of clinical trials which will provide the scientific community valuable information and lessons on the safety, efficacy and applicability of viral vectors in gene therapy.

5.4. Pediatric Use

Regarding the applicability of AAVV based **gene therapies in children** from age 0-18, there are several pediatric investigation plans (PIPs) currently being conducted [128], since there is no exclusion of young children from gene therapy per se. Indeed, hereditary genetic defects are often detected already in childhood and often times show disease symptoms early on in life [126].

The Pediatric Regulation came into force in the E.U.in January 2007, focuses on improving the health of children aged 0 to 17 years in Europeby facilitating the development and improving the availability of medicines [127].

In 2014 the results of a pediatric study were published, describing a phase I/II clinical trial in children aged 18 months to 6 years, conducted on children suffering from Mucopolysaccharidosis III A, a severe disease triggered by a defect in the sulfamidase gene. The vector is an AAVV serotype 10, containing a correct copy of the gene for sulfamidase, and injected intracerebrally. The study concluded that the intracerebral administration of AAV 10 was safe and well tolerated after 1 year in the four children studied, validating the surgical approach for direct AAV vector delivery in the brain parenchyma [128].

Spark therapeutics, which are developing a gene therapy for inherited retinal dystrophy, have tested their lead candidate, SPK-RPE65 as a single injection in the eye also in children. This medicine uses an AAV serotype 2 vector to insert the correct RPE65 gene into the eye of blinding patients. They report the loss of need for visual aid for carrying out classroom activities and better navigation skills and the improvement was reported even after a follow-up period of 2-4 years [80].

These two examples are from a series of studies regarding gene therapy with viral vector use tested in children. These results encourage companies to develop and test gene therapies in a young patient group, as to enable these to lead a normal childhood and life.

6. Summary and conclusions

The Adeno-associated virus has become a useful tool for gene transfer in the new therapeutic approach of gene therapy. The curiosity in the non-pathogenic AAV's life cycle led to basic research which, in turn, led to the discovery of its applicability in stable transduction of cells and long-term transgene expression in non-dividing cells [3].

While the areas of manufacturing and quality saw great improvements, these critical steps to produce a product for a safe and homogenously efficacious therapy are still evolving. It is also the case with the non-clinical experience data: it is an area from which we have learned a lot and will continue to learn.

Extensive non-clinical data proving the therapy's safety is necessary for ATMP's clinical trial application and for backing up a solid marketing authorization application, yet caution is advised when designing the AAVV non-clinical testing program and extrapolating pre-clinical data to humans, as it is not yet clear which animal models deliver the most relevant data. Non-clinical experience with the vector, be it *in vitro* or *in vivo*, not only contributed to the deeper understanding of AAV biology but also convinced researchers and regulating authorities to move to move into clinical use. AAV has been and is currently used in clinical trials for an array of genetic conditions with even more options for applicability through vector optimization and/or pseudotyping [8].

While this vector system's safety data is good, with no severe adverse events to date[58] , it does come with a distinct set of challenges which have to be addressed in pre-clinical and/or clinical stages of research: its potential for germline transmission, the potential for reactivation of productive infection, the impact of possible contamination with the wild-type virus and the assessment of risk for the persistent expression of the transgene product, if this feature is undesired [10].

AAV got its breakthrough on the market when alipogene tiparvovec, under the tradename of Glybera, was approved for the treatment of LPL Deficiency. Gene therapies for hemophilia and some types of inherited blindness, with extensive research data backing their claims, might potentially be on the market soon, too. The EU has thus issued the first marketing authorization for a gene therapy product in advance of the FDA and also has more guidelines on AAV. But both these regulatory agencies handling not only scientific advice on this topic but also overseeing the marketing authorizations show the willingness to communicate, cooperate and learn together with developers in order to gather more experience and information, expressed as an encouragement for open communication in different guidelines.

In conclusion, the fact that we now have a platform for long-term correction of genetic disease might revolutionize the way we treat and, ideally, cure many underserved conditions. Researchers and developers need to be encouraged to continue on this innovative path, keeping the patients in the focus of their work, many of which are waiting for this new technology to ripen for a long time, because AAV's potential to address human monogenic disease is tremendous.

References

1. Modrow, Susanne, et al. "Molekulare Virologie." (2010). Print.
2. Daya, Shyam, and Berns, Kenneth I. "Gene Therapy Using Adeno-Associated Virus Vectors." *Clinical microbiology reviews* 21.4 (2008): 583-93. Print.
3. Flotte, Terence R. "Birth of a New Therapeutic Platform: 47 Years of Adeno-Associated Virus Biology from Virus Discovery to Licensed Gene Therapy." *Molecular Therapy* 21.11 (2013): 1976-81. Print.
4. "Aav Vectors." *aavvectors.tripod.com* 2016. Web. 2 June 2016.
5. US National Library of Medicine. "What Is Gene Therapy?" *www.ghr.nlm.nih.gov* 4 October 2016. Web. 11 August 2016.
6. Nayerossadat, Nouri, Maedeh, Talebi, and Ali, Palizban Abas. "Viral and Nonviral Delivery Systems for Gene Delivery." *Advanced Biomedical Research* 1 (2012): 27. Print.
7. Ayuso, Eduard. "Manufacturing of Recombinant Adeno-Associated Viral Vectors: New Technologies Are Welcome." *Molecular therapy. Methods & clinical development* 3 (2016): 15049. Print.
8. Büning, Hildegard, et al. "Engineering the Aav Capsid to Optimize Vector–Host-Interactions." *Current opinion in pharmacology* 24 (2015): 94-104. Print.
9. abm. "Aav - Introduction | Abm Inc." *www.abmgood.com* 2016. Web. 2 June 2016.
10. European Medicines Agency. "Reflection Paper on Quality, Non-Clinical and Clinical Issues Related to the Development of Recombinant Adeno-Associated Viral Vectors". *http://ema.europa.eu* (2010). Print.
11. US Food And Drug Administration. "General Biologics Guidances - Cmc and Gmp Guidances." *www.fda.gov* 12 March 2016. Web. 19 May 2016.
12. Merten, Otto-Wilhelm, and Al-Rubeai, Mohamed. *Viral Vectors for Gene Therapy: Methods and Protocols*. Humana Press, 2011. Print.
13. US Pharmacopeial Convention. "Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests". *www.hmc.usp.org* (2009). Print.
14. World Health Organization. "Who | Quality Control." *www.who.int* 31 May 2016. Web. 5 May 2016.

15. BioMarin Pharma. "Biomarin Provides Encouraging Preliminary Data on First 8 Patients in Hemophilia a Gene Therapy Program." *investors.bmrn.com* 20 April 2016. Web. 11 May 2016.
16. World Health Organization. "The International Pharmacopoeia Fifth Edition." *www.who.int* 2015. Web. 9 May 2016.
17. Chandler, Donna KF, Volokhov, Dmitriy V, and Chizhikov, Vladimir E. "Mycoplasma-Historical Overview of Mycoplasma Testing for Production of Biologics." *American Pharmaceutical Review* 14.4 (2011): 48. Print.
18. Committee for Medicinal Products for Veterinary Use (CVMP). "Vich Gl34: Biologicals: Testing for the Detection of Mycoplasma Contamination". *www.ema.europa.eu* (2013). Print.
19. Gombold, James, et al. "Systematic Evaluation of in Vitro and in Vivo Adventitious Virus Assays for the Detection of Viral Contamination of Cell Banks and Biological Products." *Vaccine* 32.24 (2014): 2916-26. Print.
20. ICH Expert Working Group. "Viral Vafety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin Q5a (R1)". *www.ich.org* (1999). Print.
21. US Food And Drug Administration. "Guidance for Industry: Q5a Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin". *www.fda.gov* (1998). Print.
22. Peden, Keith. "Fda's Approach to Adventitious-Agent Testing of Cell Substrates and Viral Vaccines: Traditional and Novel Methods." *www.fda.gov* 7 May 2010. Web. 9 May 2016.
23. Suvarna, Kalavati. "Endotoxin Detection Methods – Where Are We Now?" *www.americanpharmaceuticalreview.com* 25 August 2015. Web. May 9 2016.
24. Ajith, Kumar. "Bio-Resource: Bacterial Endotoxin Test (Lal Test): Principle & Advantages." *http://technologyinscience.blogspot.co.at* 13 July 2012. Web. 10 May 2016.
25. Office of Regulatory Affairs. "Inspection Technical Guides - Bacterial Endotoxins/Pyrogens". *www.fda.gov* (1985). Print.
26. Council of the European Union. "European Pharmacopoeia, 8th Ed : Publ. In Accordance with the Convention on the Elaboration of a European Pharmacopoeia". *www.edqm.eu* (2013). Print.

27. Wikipedia. "Bioburden." *www.wikipedia.org* 2016. Web. 10 May 2016.
28. Taiwan Advanced Sterilization Technology Inc. "What Is Bioburden, Bioburden Testing - Ast." *http://tast.com* 2012. Web. 10 May 2016.
29. Sommer, Jürg M, et al. "Quantification of Adeno-Associated Virus Particles and Empty Capsids by Optical Density Measurement." *Molecular Therapy* 7.1 (2003): 122-28. Print.
30. Biosistemika. "Qpcr Basics - Biosistemika." *www.biosistemika.com* 2016. Web. 10 May 2016.
31. Ayuso, Eduard, et al. "Manufacturing and Characterization of a Recombinant Adeno-Associated Virus Type 8 Reference Standard Material." *Human gene therapy* 25.11 (2014): 977-87. Print.
32. Manno, Catherine S, et al. "Successful Transduction of Liver in Hemophilia by Aav-Factor Ix and Limitations Imposed by the Host Immune Response." *Nature medicine* 12.3 (2006): 342-47. Print.
33. Zhen, Zhu, et al. "Infectious Titer Assay for Adeno-Associated Virus Vectors with Sensitivity Sufficient to Detect Single Infectious Events." *Human gene therapy* 15.7 (2004): 709-15. Print.
34. Shiau, Ai-Li, Liu, Pu-Ste, and Wu, Chao-Liang. "Novel Strategy for Generation and Titration of Recombinant Adeno-Associated Virus Vectors." *Journal of virology* 79.1 (2005): 193-201. Print.
35. Haile, Lydia Asrat, et al. "Detection of Innate Immune Response Modulating Impurities in Therapeutic Proteins." *PloS one* 10.4 (2015): e0125078. Print.
36. Ayuso, E, et al. "High Aav Vector Purity Results in Serotype-and Tissue-Independent Enhancement of Transduction Efficiency." *Gene therapy* 17.4 (2010): 503-10. Print.
37. Gousseinov, Elina, Kools, Willem, and Pattnaik, Priyabrata. "Nucleic Acid Impurity Reduction in Viral Vaccine Manufacturing." *Bioprocess International* 12.2 (2014): 59-68. Print.
38. Grimm, D, et al. "Titration of Aav-2 Particles Via a Novel Capsid Elisa: Packaging of Genomes Can Limit Production of Recombinant Aav-2." *Gene therapy* 6.7 (1999). Print.

39. Chadeuf, Gilliane, et al. "Evidence for Encapsidation of Prokaryotic Sequences During Recombinant Adeno-Associated Virus Production and Their in Vivo Persistence after Vector Delivery." *Molecular Therapy* 12.4 (2005): 744-53. Print.
40. Alfayez, Ala'a. "Stability Tests for Pharmaceutical Products." *de.slideshare.net* 29 May 2012. Web. 2 June 2016.
41. Charlotte, Hoefnagel Marcel, and De, Wolf. European Medicines Agency. "Potency & Stability Testing for Atmp." *www.ema.europa.eu* 17 April 2015. Web. 2 June 2016.
42. Ellison, George, Straumfjord, Jon V, and Hummel, JP. "Buffer Capacities of Human Blood and Plasma." *Clinical chemistry* 4.6 (1958): 452-61. Print.
43. Shabushnig, John G. "Visible Particles: Regulatory and Compendial Requirements." *www.pda.org* June 2014. Web. 2 June 2016.
44. Committee for Medicinal Products for Human Use (CHMP). "Assessment Report - Glybera". *http://ema.europa.eu* (2015). Print.
45. Melchiorri, Daniela, et al. "Regulatory Evaluation of Glybera in Europe [Mdash] Two Committees, One Mission." *Nature reviews Drug discovery* 12.9 (2013): 719-19. Print.
46. Hastie, Eric, and Samulski, R Jude. "Adeno-Associated Virus at 50: A Golden Anniversary of Discovery, Research, and Gene Therapy Success—a Personal Perspective." *Human gene therapy* 26.5 (2015): 257-65. Print.
47. Walsh, Christopher E, et al. "Regulated High Level Expression of a Human Gamma-Globin Gene Introduced into Erythroid Cells by an Adeno-Associated Virus Vector." *Proceedings of the National Academy of Sciences* 89.15 (1992): 7257-61. Print.
48. Flotte, Terence R, et al. "Stable in Vivo Expression of the Cystic Fibrosis Transmembrane Conductance Regulator with an Adeno-Associated Virus Vector." *Proceedings of the National Academy of Sciences* 90.22 (1993): 10613-17. Print.
49. Nonnenmacher, Mathieu, and Weber, Thomas. "Adeno-Associated Virus 2 Infection Requires Endocytosis through the Clic/Geec Pathway." *Cell host & microbe* 10.6 (2011): 563-76. Print.
50. Uhrig, S, et al. "Successful Target Cell Transduction of Capsid-Engineered Raav Vectors Requires Clathrin-Dependent Endocytosis." *Gene therapy* 19.2 (2012): 210-18. Print.

51. Monahan, Paul E, et al. "Direct Intramuscular Injection with Recombinant Aav Vectors Results in Sustained Expression in a Dog Model of Hemophilia." *Gene therapy* 5.1 (1998): 40-49. Print.
52. Wang, Lili, et al. "Sustained Correction of Disease in Naive and Aav2-Pretreated Hemophilia B Dogs: Aav2/8-Mediated, Liver-Directed Gene Therapy." *Blood* 105.8 (2005): 3079-86. Print.
53. Marcos-Contreras, Oscar A, et al. "Sustained Correction of Fvii Deficiency in Dogs Using Aav-Mediated Expression of Zymogen Fvii." *Blood* 127.5 (2016): 565-71. Print.
54. During, MJ, et al. "In Vivo Expression of Therapeutic Human Genes for Dopamine Production in the Caudates of Mptp-Treated Monkeys Using an Aav Vector." *Gene therapy* 5.6 (1998): 820-27. Print.
55. Donsante, Anthony, et al. "Aav Vector Integration Sites in Mouse Hepatocellular Carcinoma." *Science* 317.5837 (2007): 477-77. Print.
56. Kay, Mark A. "Aav Vectors and Tumorigenicity." *Nature biotechnology* 25.10 (2007): 1111-13. Print.
57. Nathwani, Amit C, et al. "Long-Term Safety and Efficacy of Factor Ix Gene Therapy in Hemophilia B." *New England Journal of Medicine* 371.21 (2014): 1994-2004. Print.
58. Büning, Hildegard, and Schmidt, Manfred. "Adeno-Associated Vector Toxicity—to Be or Not to Be?" *Molecular Therapy* 23.11 (2015): 1673-75. Print.
59. Valdmanis, Paul N, Lisowski, Leszek, and Kay, Mark A. "Raav-Mediated Tumorigenesis: Still Unresolved after an Aav Assault." *Molecular Therapy* 20.11 (2012): 2014. Print.
60. Afione, Sandra A, et al. "In Vivo Model of Adeno-Associated Virus Vector Persistence and Rescue." *Journal of virology* 70.5 (1996): 3235-41. Print.
61. European Medicines Agency. "Guideline on Non-Clinical Testing for Inadvertent Germline Transmission of Gene Transfer Vectors". *www.ema.europa.eu* (2006). Print.
62. Arruda, Valder R, et al. "Lack of Germline Transmission of Vector Sequences Following Systemic Administration of Recombinant Aav-2 Vector in Males." *Molecular Therapy* 4.6 (2001): 586. Print.
63. Baldo, Aline, et al. "General Considerations on the Biosafety of Virus-Derived Vectors Used in Gene Therapy and Vaccination." *Current gene therapy* 13.6 (2013): 385-94. Print.

64. Russell, Stephen R. "Safety and Efficacy Study in Subjects with Leber Congenital Amaurosis." (2016). Print.
65. Dalkara, Deniz, et al. "Let There Be Light: Gene and Cell Therapy for Blindness." *Human gene therapy* 27.2 (2016): 134-47. Print.
66. Committee for the Medicinal Products for Human Use (CHMP). "Summary of Positive Opinion for Glybera". *http://ema.europa.eu* (2012). Print.
67. Kelly, Evelyn B. *Gene Therapy (Health and Medical Issues Today, 1558-7592)*. Greenwood Publishing Group, 2007. Print.
68. Wiley. "Gene Therapy Clinical Trials Worldwide." *http://www.abedia.com/wiley/* 2016. Web. 29 April 2016.
69. Wikipedia. "Cystic Fibrosis." *www.wikipedia.org* 2016. Web. 9 April 2016.
70. Warrington Jr, Kenneth H, and Herzog, Roland W. "Treatment of Human Disease by Adeno-Associated Viral Gene Transfer." *Human genetics* 119.6 (2006): 571-603. Print.
71. Moss, Richard B, et al. "Repeated Adeno-Associated Virus Serotype 2 Aerosol-Mediated Cystic Fibrosis Transmembrane Regulator Gene Transfer to the Lungs of Patients with Cystic Fibrosis: A Multicenter, Double-Blind, Placebo-Controlled Trial." *CHEST Journal* 125.2 (2004): 509-21. Print.
72. Wagner, John A, et al. "Safety and Biological Efficacy of an Adeno-Associated Virus Vector–Cystic Fibrosis Transmembrane Regulator (Aav-Cftr) in the Cystic Fibrosis Maxillary Sinus." *The Laryngoscope* 109.2 (1999): 266-74. Print.
73. Flotte, Terence R, et al. "Phase I Trial of Intranasal and Endobronchial Administration of a Recombinant Adeno-Associated Virus Serotype 2 (Raav2)-Cftr Vector in Adult Cystic Fibrosis Patients: A Two-Part Clinical Study." *Human gene therapy* 14.11 (2003): 1079-88. Print.
74. Taylor, AW. "Ocular Immune Privilege." *Eye* 23.10 (2009): 1885-89. Print.
75. Boudes, Pol F. "Gene Therapy as a New Treatment Option for Inherited Monogenic Diseases." *European journal of internal medicine* 25.1 (2014): 31-36. Print.
76. Hauswirth, William W, et al. "Treatment of Leber Congenital Amaurosis Due to Rpe65 Mutations by Ocular Subretinal Injection of Adeno-Associated Virus Gene Vector: Short-Term Results of a Phase I Trial." *Human gene therapy* 19.10 (2008): 979-90. Print.

77. Maguire, Albert M, et al. "Safety and Efficacy of Gene Transfer for Leber's Congenital Amaurosis." *New England Journal of Medicine* 358.21 (2008): 2240-48. Print.
78. Bainbridge, James WB, et al. "Effect of Gene Therapy on Visual Function in Leber's Congenital Amaurosis." *New England Journal of Medicine* 358.21 (2008): 2231-39. Print.
79. Cideciyan, Artur V, et al. "Human Rpe65 Gene Therapy for Leber Congenital Amaurosis: Persistence of Early Visual Improvements and Safety at 1 Year." *Human gene therapy* 20.9 (2009): 999-1004. Print.
80. Ledford, Heidi. "Success against Blindness Encourages Gene Therapy Researchers." *Nature* 526.7574 (2015): 487. Print.
81. National Hemophilia Foundation. "Hemophilia A." *www.hemophilia.org* 4 March 2014. Web. 3 May 2016.
82. Lusher, Jeanne M. "Hemophilia: From Plasma to Recombinant Factors". *www.hematology.org* (2008). Print.
83. Organization, World Health. "Guidelines on Viral Inactivation and Removal Procedures Intended to Assure the Viral Safety of Human Blood Plasma Products." *WHO Technical Report, Series* 924 (2004): 150-224. Print.
84. Kao, C-Y, et al. "Fix-Triple, a Gain-of-Function Factor Ix Variant, Improves Haemostasis in Mouse Models without Increased Risk of Thrombosis." *Thrombosis and haemostasis* 104.2 (2010): 355-65. Print.
85. Kay, Mark A, et al. "Evidence for Gene Transfer and Expression of Factor Ix in Haemophilia B Patients Treated with an Aav Vector." *Nature genetics* 24.3 (2000): 257-61. Print.
86. Manno, Catherine S, et al. "Aav-Mediated Factor Ix Gene Transfer to Skeletal Muscle in Patients with Severe Hemophilia B." *Blood* 101.8 (2003): 2963-72. Print.
87. Herzog, Roland W, et al. "Long-Term Correction of Canine Hemophilia B by Gene Transfer of Blood Coagulation Factor Ix Mediated by Adeno-Associated Viral Vector." *Nature medicine* 5.1 (1999): 56-63. Print.
88. Mount, Jane D, et al. "Sustained Phenotypic Correction of Hemophilia B Dogs with a Factor Ix Null Mutation by Liver-Directed Gene Therapy." *Blood* 99.8 (2002): 2670-76. Print.
89. Wikipedia. "Cytotoxic T-Cell." *www.wikipedia.org* 2016. Web. 29 April 2016.

90. Nathwani, Amit C, et al. "Adenovirus-Associated Virus Vector–Mediated Gene Transfer in Hemophilia B." *New England Journal of Medicine* 365.25 (2011): 2357-65. Print.
91. Nathwani, Amit 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* 107.7 (2006): 2653-61. Print.
92. Nagelhout, John J., Plaus, Karen L., and Nagelhout, John J. *Handbook of Anesthesia*. Maryland Heights, MO: Elsevier Saunders, 2014. Print.
93. Toromanoff, Alice, et al. "Safety and Efficacy of Regional Intravenous (Ri) Versus Intramuscular (Im) Delivery of Raav1 and Raav8 to Nonhuman Primate Skeletal Muscle." *Molecular Therapy* 16.7 (2008): 1291-99. Print.
94. US Food And Drug Administration. "Design and Analysis of Shedding Studies for Virus or Bacteria-Based Gene Therapy and Oncolytic Products - Guidance for Industry". *www.fda.gov* (2015). Print.
95. Fidler, Ben. Xconomy. "With Biomarin Data, Gene Therapy Finally Shows Impact on Hemophilia A." *www.xconomy.com* 20 April 2016. Web. April 26 2016.
96. Streetinsider. "Biomarin Pharma (Bmrn) Announces Positive Preclinical Data from Bmn 270 Phase 1/2 in Hemophilia A." *www.streetinsider.com* 20 April 2016. Web. 26 April 2016.
97. Biotechnology, Nature. "Retracing Events." *Nat Biotech* 25.9 (2007): 949-49. Print.
98. Evans, Christopher H, Ghivizzani, Steven C, and Robbins, Paul D. "Arthritis Gene Therapy's First Death." *Arthritis research & therapy* 10.3 (2008): 1. Print.
99. Tobin, John J, and Walsh, Gary. "Medical Product Regulatory Affairs." *ed. Weinheim, Germany: Wiley-VCH* (2008). Print.
100. Fontaine, N, and Reynders, D. "Directive 2001/83/Ec of the European Parliament and of the Council of 6 November 2001 on the Community Code Relating to Medicinal Products for Human Use." *Official Journal of the European Communities L* 311 (2001): 67-128. Print.
101. Committee for Proprietary Medicinal Products (CPMP). "Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products". *http://ema.europa.eu* (2001). Print.

102. Center for Biologics Evaluation and Research. "Cellular & Gene Therapy Guidances." *www.fda.gov* 9 September 2016. Web. 15 September 2016.
103. Council of the European Union. "Regulation (Ec) No 297/95". *www.ec.europa.eu* (1995). Print.
104. Human Medicines Research and Development Support. "European Medicines Agency Guidance for Applicants Seeking Scientific Advice and Protocol Assistance". *www.ema.europa.eu* (2014). Print.
105. US Food And Drug Administration. "Fda Approves First-of-Its-Kind Product for the Treatment of Melanoma." *www.fda.gov* 27 October 2015. Web. Oct 2 2016.
106. FDA News. "Uniqure Won't Seek U.S. Regulatory Approval for Glybera Gene Therapy Program." *www.fdanews.com* 9 December 2015. Web. October 2 2016.
107. European Medicines Agency. "Chmp - Scientific Advice Working Party." *www.ema.europa.eu* 2016. Web. October 2 2016.
108. Antunes, M, and Pottering, H. "Regulation (Ec) No 1394/2007 of the European Parliament and of the Council of 13 November 2007 on Advanced Therapy Medicinal Products and Amending Directive 2001/83/Ec and Regulation (Ec) No 726/2004." *Journal of European Union L* 324 (2007): 121-37. Print.
109. European Medicines Agency. "Cat: Overview." *www.ema.europa.eu* 2016. Web. October 2 2016.
110. European Medicines Agency. "Multidisciplinary: Gene Therapy." *www.ema.europa.eu* 2016. Web. June 2 2016.
111. European Medicines Agency. "Biologicals - Quality, Preclinical and Clinical Aspects of Gene Therapy Medicinal Products". *www.ema.europa.eu* (2001). Print.
112. US Food And Drug Administration. "Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy". *www.fda.gov* (1998). Print.
113. US Food And Drug Administration. "Guidance for Industry: Gene Therapy Clinical Trials and Observing Subjects for Delayed Adverse Events". *www.fda.gov* (2006). Print.
114. Wiklund, Fredrik. "Celladon Receives Breakthrough Therapy Designation from Fda for Mydicar." *ir.celladon.com* 10 April 2014. Web. 2 October 2016.

115. US Food And Drug Administration. "Developing Products for Rare Diseases and Conditions." *www.fda.gov* 22 September 2016. Web. 2 June 2016.
116. European Medicines Agency. "Human Regulatory - Prime: Priority Medicines." *www.ema.europa.eu* 2016. Web. 2 October 2016.
117. European Medicines Agency. "Adaptive Pathways: Key Learnings and Next Steps." *www.ema.europa.eu* 3 August 2016. Web. 2 October 2016.
118. European Medicines Agency. "Launch of Prime – Paving the Way for Promising Medicines for Patients." *www.ema.europa.eu* 3 July 2016. Web. 2 October 2016.
119. US Food And Drug Administration. "Orphan Products Grants Program." *www.fda.gov* 13 January 2016. Web. 12 March 2016.
120. European Medicines Agency. "Human Regulatory - Orphan Designation." *www.ema.europa.eu* 2016. Web. 12 March 2016.
121. Orphanet. "Orphanet: List of Orphan Designations." *www.orpha.net* 15 January 2016. Web. 12 March 2016.
122. BioMarin Pharma. "Biomarin Receives Orphan Drug Designation from Fda for First Aav-Factor Viii Gene Therapy, Bmn 270, for Patients with Hemophilia A." *www.bmrn.com* 1 March 2016. Web. 12 March 2016.
123. BioMarin Pharma. "Biomarin Receives European Orphan Drug Designation for Bmn 270, First Investigational Aav-Factor Viii Gene Therapy for Patients with Hemophilia A." *investors.bmrn.com* 24 March 2016. Web. 2 October 2016.
124. Orlet, Chris. "Agtc Gets Eu Orphan Drug Status for Gene Therapy for Achromatopsia | Market Scope." *www.market-scope.com* 30 October 2015. Web. 2 October 2016.
125. AGTC. "Agtc Announces U.S. Fda Orphan Drug Designation for Gene Therapy to Treat Achromatopsia." *ir.agtc.com* 23 November 2015. Web. 12 March 2016.
126. Wikipedia. Wikipedia. "Genetic Disorder." *www.wikipedia.org* 2016. Web. October 3 2016.
127. European Medicines Agency. "Paediatric Medicine - Paediatric Regulation." *www.ema.europa.eu* 2016. Web. 12 March 2016.
128. Tardieu, Marc, et al. "Intracerebral Administration of Adeno-Associated Viral Vector Serotype Rh. 10 Carrying Human Sgsh and Sumf1 Cdnas in Children with

Mucopolysaccharidosis Type Iiia Disease: Results of a Phase I/Ii Trial." *Human gene therapy* 25.6 (2014): 506-16. Print.