CLINICAL
INVESTIGATION

# Gene therapy in neurology: review of ongoing clinical trials

Gene therapy has entered into its third decade since the first human clinical trial in 1990. It is an expanding field and the use of sequence-targeted regulatory molecules may pave one path to the personalized therapy of genetic disorders. However, gene therapy may not only help patients with monogenic disorders, but may also establish a gene reservoir of the theraputic protein in polygenic, multifactorial disorders and help to destroy malignant tumor cells. Several clinical trials for neurologic syndromes appear quite promising. The field of gene therapy has been driven by genomic technologies, including identifying disease-causing genes and mutations, design of genomic interacting elements to regulate transcription and splicing of specific precursor mRNAs and use of novel noncoding regulatory RNAs. This review covers gene therapy strategies to ameliorate neurologic syndromes of different etiologies, including muscular dystrophies, lysosomal storage diseases, amyloidosis, Alzheimer's and Parkinson's disease, amyotrophic lateral sclerosis, pain and brain tumors. The advances in clinical arenas, the successes in this field and the remaining obstacles are highlighted.

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The field of gene therapy developed approximately 35 years ago and has had many setbacks along the way to its most recent successes. Although the first gene was successfully delivered into mammalian cells in 1977 [1], *in vitro* gene transfer proof-of-concept studies were not widely performed until the 1980s. The first gene therapy was approved to treat severe childhood immunodeficiency (SCID) in 1990. The effects were only temporary, but successful [2]. Unfortunately, in 1999 a patient suffering from ornithine transcarboxylase deficiency died from organ failure after gene therapy [201]. The patient suffered an immune response against the virus that carried the new gene into his cells. In 2000, Fischer *et al.* cured children with SCID by applying a retroviral vector, but later four of the children developed leukaemia [3]. Therefore, due to safety concerns, the US FDA placed a temporary halt on all gene therapy trials using retroviral vectors in blood stem cells. These two early unsuccessful events discouraged many researchers from the continuation of gene therapeutical research.

In 2006 two patients suffering from metastatic melanoma were successfully treated with genetically altered killer T cells that attacked their cancerous cells, thus ushering in a new era in gene therapy [4]. In the same year gene-based immunotherapy was succesful in the treatment of HIV using lentiviral vector for delivery of an antisense gene against the HIV envelope *ENV* gene. [5]. Then, 3 years later in 2009, researchers succeeded in the treatment of adrenoleukodys-trophy using a vector derived from HIV to deliver the gene *ABCD1* gene, which is involved in the import and anchoring of the very long-chain fatty acid CoA

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synthetase in the peroxisomes [6]. Since 2006, the number of gene therapies has increased significantly. According to the FDA, the number of ongoing gene therapy clinical trials has increased from 1064 to 3398 between 2006 and 2011 [202]. Although gene therapy is yet to come of age, there is no doubt that the therapeutic application of these therapies is only a matter of time. This review highlights the most up-to-date data from gene therapy clinical trials of the nervous system and muscle. These treatment modalities include various forms of gene therapies.

## General aspects of gene therapy

Gene therapy comprises treatment modalities designed to correct or neutralize specific underlying molecular defects (or their deleterious consequences) occurring in the particular genetic disease. These therapies can be categorized into different categories of diseases of the nervous system and musculoskeletal system. Gene therapy can be used to:

- Treat genetic diseases;

- Establish stable gene reservoirs as sources of therapeutic proteins in multifactorial diseases;

- Increase the sensitivity of tumor cells to drugs;

- Destroy malignant cells in neoplasms;

- Produce DNA-based vaccines.

For the aforementioned goals, DNA- and RNA-based methods can be appropriate. DNA-based therapies comprise:

- Gene replacement (GR);

- Gene transfer (to upregulate therapeutic protein);

- Gene editing (to correct the defected gene) approaches.

The possible RNA-modulating treatment methods are:

- Exon skipping by antisense oligonucleotides;

- Mutant RNA removal;

- Inactivation of the mutant mRNA by RNAi;

- Destruction of the mutant mRNA by ribozymes.

The aforementioned treatment strategies are currently in the preclinical and clinical development phase. Aiming at promising results by gene therapies, one has to be mindful of certain biological characteristics of the target cells and tissues. In the case of the CNS, the blood–brain barrier on one hand hinders access of hematogenously disseminated vectors or gene products originating from depots in transduced peripheral tissues, to cells of the CNS [7]. On the other hand, the blood–brain barrier might provide an immunologically privileged status for the CNS that can be useful in mitigating the immune-mediated elimination of cells after transduction with viral vectors. Mature, differentiated neurons are postmitotic, therefore retroviral vectors are not suitable for gene delivery. Vectors that do not integrate in the host genome are diluted, as with adenovirus (AV). Neurons and glial cells form contacts with eachother, which can serve as a transfer pathway of the introduced transgene, its protein product, or both. Synaptic contacts between neurons present another potential route for transfer of vectors from one neuron to another, particularly in the case of viral vectors [8]. Regional heterogeneity of neurons provides an opportunity to use particular gene promoter/enhancer units that are specific for a particular neuronal group. The neuronal heterogeneity could also entail a differential transducibility or transfectability of different neuronal populations by specific vectors [9].

There are certain specific cellular features in skeletal muscle that are relevant to gene therapy. Similarly to mature neurons, mature muscle fibers are postmitotic. Skeletal muscle fibers are multinucleated, which implies that in the case of a nondiffusable protein product of a gene, many myonuclei must take up the therapeutic vector in order to have a diffuse distribution of the therapeutic protein throughout the muscle fiber. Skeletal muscle fibers are surrounded by dormant myogenic satellite cells, which can potentially fuse into the parent fiber and deliver therapeutic genes into that cell. Factors that can promote such fusion of satellite cells into the host muscle fibers include regeneration and work hypertrophy [9].

## Gene therapy of genetic disorders

In the treatment of genetic diseases there are four sequential levels that can be targeted by therapeutic interventions that may be specific or nonspecific for a particular disease:

- DNA level: the gene defect;

- RNA level: pre-mRNA or mRNA molecule;

- Protein level: replacement of missing protein;

- Phenotypic level: pathological cellular and tissue alterations th at result in clinical signs and symptoms.

Therapeutic and preventative approaches may be directed at any of the four domains cited above.

Some therapies may target more than one of these domains. The scope of this article is gene therapeutical interventions in clinical trials of genetic and

nongenetic neurological disorders.

## DNA-modulating therapies

### ■ GR

This procedure entails the introduction of functionally adequate alleles (usually cDNAs) into tissues that are most affected by the deleterious effects of the gene defect. This approach is more suitable for monogenic diseases inherited in a recessive trait, due to a single defective gene. GR may be employed *in vivo*, that is, introduction of the therapeutic gene directly into the affected cell tissues, or *ex vivo*. During the latter approach, the diseased host cells are first removed from the body, then subsequently transfected or transduced with the therapeutic gene and finally introduced back into the host.

In order to achieve good results by GR, several items should be optimized in preclinical experiments. The determination of the most appropriate transferable gene, promoter, vector and route of administration is crucial. The transferable gene is usually only the coding sequence of a gene (cDNA). However, in some instances, the therapeutic cDNA is too large for the capacity of the employed gene vector. In this case a truncated cDNA may be used that could still generate a functionally adequate, albeit not perfect, protein [9]. Another issue is the possible immunogenicity of the new protein generated by the transferred gene, to which the host's immune system may have intolerance. The promoter controls the expression of a therapeutic gene in a 'cassette'. It has several properties that include efficiency and specificity for a given cell and low susceptibility for inactivation. The ideal promoter would satisfy these criteria, but in many instances the natural promoter is not suitable for this role. However, the risk of late promoter inactivation in the context of episomal vectors appears to be a major problem. For efficient GR, various vectors have been used along the years, namely nonviral and viral vectors. An ideal gene vector bears the following characteristics: efficiency for a given cell type, lack of immunogenicity, lack of nonimmune-mediated toxicity, harmless integration into the host genome, relatively easy and cost-effective production of high vector-particle number per volume under GMP conditions [9]. As a matter of fact, none of the used vectors currently conform perfectly to all of these requirements. Nonviral vectors include circular or supercoiled plasmids, liposomes, protein ligands and nanoparticles. Efficiency of entry of these nonviral vectors to muscle fibers *in vivo* is relatively low after direct injection [10]. The only possible exception is the plasmid vector, which transfects very few muscle fibers when injected directly into muscle but seems to transfect a significant number of muscle

fibers if injected intra-arterially [11]. The risk of immunogenicity in the case of plasmid DNA is minimal. Electroporation and sonoporation after intramuscular administration of the plasmid containing the therapeutic gene also appear to improve the plasmid uptake to muscle fibers [10,12]. Recently, a new 'bionic chip' has been developed to increase the trasfection efficacy [101]. It contains a microelectroporation device that combines microarrays of oligonucleotides, microfluidic channels and electroporation for cell transfection and high-throughput screening applications. The most efficient and widely used vectors for therapeutic gene delivery have been genetically modified viruses. The most commonly used viral vectors for CNS diseases are AV, adeno-associated virus (AAV) and herpes simplex virus 1 [9]. AV has many attractive features as a therapeutic gene vector, including the ability to infect both mitotic and postmitotic cells, easy production of high titer during cultivation, easy purification, potentially large insert capacity, and practically no oncogenicity. However, AV proteins are highly immunogenic and the AV does not integrate into the host genome. Furthermore, the density of primary (coxackie and AV [CAR]) and secondary receptors (integrin dimer) on the surface of mature cells may be scarce [12]. This is particularly true for mature muscle fibers. The antigenic AV proteins induce both cellular and humoral immune responses in immunocompetent hosts, which greatly limit their applicability. The episomal existence seems to compromise the longevity of the AV vector, possibly as a result of promoter inactivation (by methylation) or by endonuclease-related damage to the expression cassette [13]. The AAV is presently the best candidate as a therapeutic gene vector for the CNS and musculoskeletal diseases since, unlike AV, it transduces mature cells well and a significant proportion seems to integrate into the host genome [14]. A relatively small insert capacity (maximum 5 kb) and difficult cultivation are the major disadvantages of AAV. The route of administration can be a critically important issue regarding skeletal muscle and CNS as well. Multiple intramuscular injections employed in preclinical experiments have little practical usefulness, since the spread of the vector from the injection site is very limited. Additionally, some muscles (i.e., diaphragm and intercostals) are not easily accessible. The intravascular application is deemed to be the ideal route of administration into skeletal muscle. The intravenous administration is not a suitable approach, since most vectors through this route end up in the liver [15]. Systemic intra-arterial injection requires an invasive preparation in which too high particle number/ml/blood volume is probably not safe. Consequently, the regional intra-arterial

route seems to be the best choice of administration route. In fact, in rats it has been shown that an efficient transduction of leg muscles could be achieved after injection of AV vectors expressing β-galactosidase into the femoral artery [16]. So far, the disadvantage of this approach is the requirement of an unusually high volume of injectate, which can cause edema and ischemic damage to the muscle. The intravascular administration of the viral vectors in hematological disorders is much more efficient [17].

■ Ongoing or recently published clinical trials applying GR for the treatment of monogenic neurologic disorders

α-sarcoglycan deficiency

α-sarcoglycan is a member of the dystrophin-associated proteins and its deficiency results in limb girdle type muscular dystrophy 2D. The therapeutic α-sarcoglycan gene (*SGCA*) with muscle specific promoter was introduced by the researchers into the muscle fibers using an AAV1 vector. Patients (n = 3) were administered with intramuscular injection of *SGCA* into the extensor digitorum brevis muscle on one side; as a control the same muscle was injected with saline on the other side of the same patient [18]. Patients were treated for 3 days with methylprednisolone to inhibit the immunological reactions. No adverse events were reported. The *SGCA* gene expression increased four- to five-times compared with the control side by week 6 (two patients) and at 3 months (one patient). Not only did the *SGCA* expression improve, but the whole sarcoglycan complex was restored. On the second week after administration, AAV1-neutralizing antibodies appeared. Currently, a trial is recruiting participants to evaluate the safety and effectiveness of rAAV1. tMCK.human-α-sarcoglycan transfection in treating children and adults with limb girdle muscular dystrophy type 2D [203].

γ-sarcoglycan deficiency

γ-sarcoglycanopathy, or limb girdle muscular dystrophy type 2C, is an untreatable disease caused by autosomal recessively inherited mutations of the γ-sarcoglycan gene (*SGCG*). The γ-sarcoglycan is a member of the dystrophin-associated proteins, similarly to the abovementioned α sarcoglycan. In the Phase I/II trial by Genethon, nine nonambulatory patients with del525T homozygous mutation of the *SGCG* and without γ-sarcoglycan immunostaining on muscle biopsy were divided into three equal groups to receive three escalating doses of an AAV1 vector expressing the human *SGCG* gene (under the control of the desmin promoter), by local injection into the *extensor carpi radialis* muscle [19,204]. The therapy was well tolerated and no serious adverse events occurred during 6 months of follow-up. After 30 days, immunohistochemical analysis of injected-muscle biopsy specimens in all three of the patients who received the highest dose showed 4.7–10.5% positively stained fibres. In one patient, γ-sarcoglycan protein was detected by western blot. For two other patients who received the low and intermediate doses, discrete levels of γ-sarcoglycan expression were also detectable. All patients became AAV1 seropositive and one subject developed a cytotoxic response to the AAV1 capsid. These findings represent a proof-of-concept for SCGC-replacement therapy in patients with limb girdle muscular dystrophy type 2C. Further research will be required to achieve widespread vector delivery, using locoregional [20] or, ultimately, systemic delivery of AAV–SCGC vectors.

Pompe disease

Pompe disease (also known as lysosomal storage disease) is an inherited condition of acid α-glucosidase (GAA) deficiency resulting in lysosomal accumulation of glycogen in all tissues. Glycogen accumulation results in muscle dysfunction and profound muscle weakness. Pompe disease manifestations can vary a great deal, with the most severely affected patients presenting with cardiorespiratory failure, often fatal in the first 2 years of life. Enzyme-replacement therapy (ERT) of the missing GAA may help to reduce the clinical symptoms. However, it has become apparent that not all patients react to ERT in the same way. Some patients have substantial improvement following ERT, while others develop chronic disability reminiscent of the later onset disease. In order to improve the current clinical outcomes in Pompe patients with diminished clinical response to ERT, researchers have developed an innovative way to introduce the normal GAA gene into muscle cells with the expectation that the GAA protein will be produced at levels sufficient to reduce glycogen accumulation. An ongoing Phase I/II study is evaluating the safety of the experimental gene transfer procedure in individuals with GAA deficiency after diaphragmal delivery. The study will also determine the most appropriate dose to achieve improvement as measured by respiratory function parameters. While a single treatment would be ideal, the complex nature of these diseases may unavoidably limit the efficacy of single therapies. In order to treat lysosomal storage disorders more efficiently, a shift in focus towards a combination therapy is necessary [205].

Batten disease: late infantile neuronal ceroid lipofuscinosis

Batten disease is a lysosomal storage disease affecting the CNS, with generally recessive inheritance. Progressive loss of vision, decreasing cognitive and motor skills, epileptic seizures and premature death are the most characteristic clinical symptoms of the disease.

Worgall *et al.* first treated children suffering from late infantile neuronal ceroid lipofuscinosis (LINCL) with gene therapy [21]. In order to introduce the human *CLN2* cDNA, a replication deficient AAV2 CU h-CLN2 was applied and the drug was directly injected into the child's brain [21]. In the treated patients, the neurologic rating scale, which was the primary outcome variable, demonstrated a significantly reduced rate of decline compared with control subjects. Four of the ten subjects developed a mild, mostly transient, humoral response to the vector. The follow-up study is currently ongoing [206] and another trial is also recruiting patients. In this investigation, similarly to the previous study, the investigators propose to administer a biologic drug by direct gene transfer into the brain and assess its safety on children with a fatal genetic disease of the CNS. The experimental drug tested during this trial is AAVrh10CUCLN2 [207].

### Sanfilippo A syndrome

Mucopolysaccharidosis III (Sanfilippo syndrome) is a lysosomal storage disorder and belongs to the group of mucopolysaccharidoses. Mucopolysaccharidosis III is caused by the deficiency of one of the four enzymes catalyzing the degradation of the glycosaminoglycan heparan sulfate. Sanfilippo A syndrome is caused by the defect of the *SGSH* gene, which encodes a lysosomal enzyme, *N*-sulfoglucosamine sulfohydrolase (sulfamidase). An open-label, single-arm, monocentric, Phase I/II clinical study is presently recruiting patients (n = 4) to evaluate the tolerance and safety of intracerebral administration of AAV viral vector serotype 10 carrying the human *SGSH* and *SUMF1* cDNAs [208]. The treatment plan consists of a direct injection of the investigational medicinal product SAF-301 to both sides of the brain through six image-guided tracks, with two deposits per track, during a single neurosurgical session. The primary objective is to assess the tolerance and safety associated with the proposed treatment through a 1-year follow-up. The secondary objective is to collect data to define exploratory tests that could become evaluation criteria for further clinical Phase III efficacy studies.

### RNA-modulating therapies

RNA-modulating therapeutics provide a powerful tool for targeted modulation of gene expression by the use of single-stranded RNA-based antisense oligonucleotides (AONs). RNAi and AONs downregulate gene expression by inducing enzyme-dependent degradation of targeted mRNA. Steric-blocking oligonucleotides block the access of cellular machinery to pre-mRNA and mRNA without the degradation of RNA. Through this mechanism the oligonucleotides can redirect alternative splicing, repair defective RNA, restore protein production or downregulate gene expression. AONs are short, synthetic and highly sequence-specific nucleic acids designed to bind to specific messenger RNAs in order to modulate splicing patterns or inhibit protein translation. The development of an AON-based approach started in the late 1970s when oligonucleotides were used as tools to downregulate the expression of specific genes [22]. AONs can be injected intramuscularly, intravenously, intraperitoneally or subcutaneously, or they can be administered orally. Intramuscular injection results in high skipping levels; however, repeated injection of all muscles is deemed to be impractical. For the heart and diaphragm this is impossible. Since AONs are relatively big molecules, only a small amount will reach the muscles from the intestinal lumen, therefore oral treatment is not a viable option. Additionally after intravenous, intraperitoneal and subcutaneous injection, a relatively small amount of AONs will exit the blood vessels and pass the cell membrane. Due to the fact that AONs are injected directly into the bloodstream, short-term skipping levels are highest after intravenous injection. A high peak dose of AONs might easily lead to detrimental effects and therefore subcutaneous injection is the safer option.

Multiple AONs are in clinical trials and only one has been approved by the FDA so far – Vitravene® (fomivirsen) – for the treatment of cytomegalovirus (CMV) infection. Fomivirsen inhibits the replication of human CMV by binding to the complementary sequence of the mRNA transcribed from the major immediate-early transcriptional unit of CMV. Binding of fomivirsen in this region inhibits the synthesis of proteins that are essential for production of infectious CMV [23]. More recently, research on aptamers for the treatment of age-related macular degeneration has led to the development of Macugen® (pegaptanib) [24]. Pegaptanib is a modified oligonucleotide that binds with high specificity and affinity to extracellular VEGF165, inhibiting its activity. The selective inhibition with pegaptanib proved as effective at suppressing pathological neovascularisation as pan-VEGF inhibition, however pegaptanib did not affect the normal vasculature whereas pan-VEGF inhibition did.

Five further AONs are currently being tested in

clinical trials: phosphorothioate, 2′-O-methyl RNA, 2′-O-methoxyethyl RNA, 2′, 4′-bridged nucleic acid/locked nucleic acid and the phosphorodiamidate morpholino oligomer (PMO) [25].

The AON compounds are designed to interfere with splicing in order to:

- Induce exon skipping;

- Enhance exon inclusion;

- Correct splicing mutations;

- Remove mutant RNA or protein domains;

- Block RNA expression.

Pre-mRNA targeting is currently used both as a research tool; for example, in models for spinal motorneuron disease, and in clinical trials for Duchenne muscular dystrophy (DMD) and amyotrophic lateral sclerosis. AONs are particularly promising in relation to brain research as modified AONs are taken up extremely fast into neurons and glial cells with long residence times [26].

RNAi is a natural process of gene silencing that occurs in organisms ranging from plants to mammals. siRNAs are molecules that mediate RNAi. These therapeutic siRNAs target the cause of diseases by effectively silencing specific mRNAs, thereby preventing disease-causing proteins from being produced.

Additional techniques include trans-splicing by special ribozymes of the primary transcripts [27], which can be used to shorten the deleterious trinucleotide repeats that occur, for example, in several CNS diseases such as Huntington's disease and myotonic dystrophy.

### ■ Exon skipping by antisense oligonucleotides: correcting the reading frame

Exon skipping is a mutation-specific therapy that provides the possibility of personalized treatment and can maintain the original tissue-specific gene regulation. They represent promising therapeutic tools for many disorders, in particular DMD [28]. Clinical trials have recently been conducted for DMD, where exon skipping were used to achieve beneficial therapeutic outcomes. However, despite promising early results, the therapeutic application of AON has proved to be difficult and its integration into the standard of care has been a concern [29].

AON-mediated exon-skipping strategies for DMD aim at removing the mutated exon alone or together with additional exons to restore the reading frame. As a consequence, it induces the expression of a shortened but functionally active form of dystrophin resulting in a Becker muscular dystrophy-like phenotype. According to the Leiden muscular dystrophy database, exon skipping is potentially applicable to approximately 83% of all DMD patients if single- and double-exon skipping of deletions, small mutations and duplications can be achieved [30]. Fortunately, the majority of deletions cluster into hotspot regions between exons 43 and 53, suggesting that skipping of the same group of exons are applicable to large groups of patients. The most notable example is exon 51 skipping, which is detected in 13% of all patients (correct deletions of exons 50, 52, 45–50, 48–50 and 49–50). For this reason, it has been targeted in two Phase I clinical trials conducted by Prosensa together with GlaxoSmithKline (GSK) and AVI-Biopharm.

Prosensa selected a 20-mer antisense oligonucleotide (AON) of 2'OMe phosphorothiate AON targeting exon 51 (PRO051). In the first Phase I trial, four DMD patients were injected locally in the tibialis anterior muscle with a single dose of 0.8 mg PRO051. Muscle biopsy was analyzed for each patient 4 weeks after the injection and revealed a restoration of dystrophin in the vast majority of muscle fibers at levels varying between 17 and 35%, in the absence of treatment-related adverse effect [31]. In Prosensa's next trial, PRO051 was systemically administered weekly by subcutaneous injections for 5 weeks in 12 patients. Four dose levels were selected (0.5, 2.0, 4.0 and 6.0 mg/kg), and each dose cohort consisted of three patients [32]. New dystrophin expression was observed between approximately 60 and 100% of muscle fibers in ten out of the 12 patients, as measured in post-treatment biopsy, which increased in a dose-dependent manner up to 15.6% of the expression in healthy muscle. All patients entered a 12-week open-label extension phase, during which they all received PRO051 at a dose of 6.0 mg/kg/week. After the 12-week extension phase, there was a mean (±SD) improvement of 35.2 ± 28.7 m (from the baseline of 384 ± 121 m) on the 6-min walk test (6MWT). The most common adverse events were irritation at the administration site and mild proteinuria and increased urinary $\alpha(1)$-microglobulin levels were detected during the extension phase. The terminal half-life of PRO051 was 29 days. PRO051 induced detectable, specific exon 51 skipping at doses of 2.0 mg/kg or more. The 48-week follow-up data of this trial detected increasing amounts of muscle protein dystrophin in muscle fibers and a further increased 6MWT distance [32]. No serious adverse events or unwanted immune responses occured, although some participants had low-level excretion of protein in the urine, which required further monitoring. Prosensa has partnered with GSK for the further clinical development of PRO051/GSK2402968. A Phase III study

started early 2011 and is currently recruiting patients [209]. Several other studies are ongoing, such as a Phase II study comparing two doses [210], a Phase II dose-ranging study [211], a safety and pharmacokinetics study in nonambulatory male DMD patients [212] and an open-label study for patients that have previously participated in such studies [213].

AVI Biopharma selected a PMO AONs based on preclinical studies [34,35]. The 30-mer PMO skipping exon 51 (AVI-4658 or eteplirsen) was injected unilaterally into the *extensor digitorum brevis* muscles in seven patients, in a single-blinded, dose-escalation protocol that included a placebo-control group (saline administered to the contralateral *extensor digitorum brevis* muscle) [214]. Results from this trial demonstrated that PMO oligonucleotides were well tolerated by all patients and dystrophin protein were expressed by up to 42% of the normal levels in dystrophin-positive fibers of patients treated with the higher dose of 0.9 mg [36,37].

In another trial, eteplirsen was adminstered intravenously to 19 boys suffering from DMD [215]. The patients had mutations in the dystrophin gene that can potentially benefit from skipping of exon 51. The treatment lasted for 12 weeks and the following doses were applied: 0.5, 1.0, 2.0, 4.0 10.0 and 20.0 mg/kg per week. Participants tolerated all doses well. Muscle biopsies were performed at baseline and at 14 weeks, patients were followed up for a total of 26 weeks. Investigators found that seven patients responded to treatment doses higher than 2 mg/kg. Mean dystrophin fluorescence intensity changed from 8.9 to 16.4%. Three patients with the greatest responses to treatment had 15, 21 and 55% dystrophin-positive fibers after treatment. These findings were also confirmed with western blot. The dystrophin-associated proteins such as α-sarcoglycan and nNOS were also restored. Functional benefit will be assessed in future studies that employ higher doses of eteplirsen for longer treatment durations [38]. AVI-Biopharm has an active study designed to assess the efficacy, safety, tolerability and pharmacokinetics of eteplirsen in both 50.0 and 30.0 mg/kg/doses administered weekly over 24 weeks in subjects diagnosed with DMD [216].

Besides exon 51, other exons are also targeted by exon-skipping techniques. Presently, Prosensa Therapeutics runs a Phase I/II trial in DMD targeting the exon 44 [217]. The skipping of exons 43, 52, 53 and 55 with PRO043, 052, 053 and 055 are in the company's pipeline. At the same time, Genethon is conducting a trial skipping the exon 53 by another method (pre-U7.53) [218].

■ **Inactivation of the mutant mRNA by AONs**

Polyglutamine (polyQ) diseases are a group of disorders caused by CAG triplet repeat expansions in the coding region of the genome. The proteins responsible for symptoms in these polyQ diseases are very different, but in each case the expanded stretch of glutamines results in a toxic gain-of-function of the protein and this leads to neurodegeneration. There is an inverse correlation of disease onset and polyQ length in the protein, that is, the longer the CAG repeat, the earlier the age of onset of the disease [39]. Protein aggregates are found in the nucleus and cytoplasm of cells, indicating that protein misfolding is a common feature of these disorders.

Evers *et al.* showed the first evidence of a specific reduction of mutant huntigtin, ataxin-1 and -3, and atrophin-1 transcript levels applying AON that recognizes pure CAG repeat stretches, suggesting that a single AON is potentially applicable to polyQ neurodegenerative diseases with an expanded pure-CAG repeat [40]. Prosensa has developed a lead compound that reduces cellular levels of aberrantly expanded huntingtin mRNA and mutant huntingtin protein. It is now being further tested in preclinical experiments. Due to the similar therapeutic approach, the same compound can also be used to treat other CAG trinucleotide repeat expansion diseases (TREDs), such as various spinocerebellar ataxia types, Huntigton's and Kennedy's diseases and Huntington's disease-like 2 [219]. Another trinucletoide repeat disease is myotonic dystrophy, in which the underlying molecular defect is the CUG repeat expansion. Prosensa has developed lead compounds that are now in preclinical testing to treat this disease.

An RNA-based therapeutic approach to knockdown gene or protein expression is the application of AONs. In 20% of familial ALS patients, the disease is caused by a mutation in SOD1 [41]. Continuous intraventricular infusion of AONs successfully downregulated the SOD1 mRNA and protein levels in the brain and significantly slowed disease progression in an animal model of ALS [42]. A Phase I clinical trial is currently ongoing in ALS patients with SOD1 mutations and results are expected this year [220].

■ **Inactivation of the mutant mRNA by RNAi**

In neurology, the first human trial using RNAi is conducted by Alnylam in transthyretin (TTR)-mediated amyloidosis [221]. This is a Phase I trial in which the ALN-TTR01 is administered intravenously. ALN-TTR01 blocks pathogenic accumulations of mutant TTR in peripheral tissues (95% reduction of V30M hTTR deposition). Delivery by next-generation liponanoparticle in 31 patients or carriers with familiar amyloid neuropathy or cardiomyopathy were enrolled

into the study. The serum TTR level is monitored during the trial. ALN-TTR01 targets the *transthyretin* gene, a gene predominantly expressed in the liver and the normal function of which is to facilitate the transport of vitamin A and thyroxin, but when mutated is prone to misfolding and deposition into a number of tissues as amyloid fibrils. This causes damage to the surrounding tissues and typically first manifests as either predominantly familiar amyloid polyneuropathy or cardiomyopathy depending on the genotype. ALN-TTR02 comprises a siRNA formulated in a proprietary second-generation lipid nanoparticle. Alnylam recently filed a clinical trial application for ALN-TTR02 to conduct a Phase I trial in the UK as a randomized, single-blinded, single ascending-dose study enrolling 32 healthy volunteers. The primary objective of the study is to evaluate the safety and tolerability of a single dose of ALN-TTR02, in subjects being enrolled into five sequential dose cohorts ranging from 0.01 to 0.50 mg/kg. Secondary objectives include assessment of pharmacodynamic activity of ALN-TTR02 as measured by serum TTR levels. Alnylam plans to begin enrollment of this clinical trial in the first half of 2012 and expects to report data in the third quarter of 2012. In addition, in the second half of 2012 Alnylam plans to start a Phase II multiple-dose study of ALN-TTR02 in patients with transthyretin-related hereditery amyloidosis and, provided that early phase clinical trials produce positive results, expects to start a pivotal trial for ALN-TTR02 in 2013.

### ■ Translational manipulation

In situations when a primary stop codon caused by a nonsense mutation leads to a truncated and unstable protein, corrupting the ability of the ribosomes to recognize stop codons could be beneficial. Aminoglycosides have such affect on ribosomes as evidenced by the fact that, systemic administration of gentamycin to mdx mice model produced full-length dystrophin in muscle fibers, presumably by the 'read-through' phenomenon [43]. However, it seems that in humans, the dose of gentamycin required, for such an effect and generating large amounts of nontruncated dystrophin would probably require a prohibitively toxic dose of gentamycin and prolonged administration. PTC Therapeutics developed ataluren (PTC124), a drug that allows the ribosome to ignore the premature stop signal and continue translation of the mRNA, resulting in formation of functioning protein in patients with genetic disorders due to a nonsense mutation. Ataluren does not cause the ribosome to read through the normal stop signal. It was investigated for use in patients with nonsense mutation DMD, Becker muscular dystrophy and cystic fibrosis (CF). The compound

failed at the Phase IIb trial in March 2010 conducted in nonsense mutation DMD patients [222]. The primary end point – the 6MWT – did not increase significantly during the 48-week treatment period. Presently, an open-label trial is ongoing for patients with nonsense mutation dystrophinopathy who received ataluren in a prior PTC-sponsored study at a US clinical trial site. This trial will be conducted at sites in the USA and will evaluate the long-term safety of ataluren, as determined by adverse events and laboratory abnormalities [223]. However, the Phase IIa clinical trials of ataluren in pediatric and adult patients with nonsense mutation CF showed that administration of ataluren resulted in production of functional CFTR protein and statistically significant improvements in CFTR chloride channel function. Ataluren treatment was associated with significant reductions in cough frequency and trends toward improvement in pulmonary function tests [224]. The Phase III trial is currently ongoing in CF indication [225].

## Gene therapy for complex, polygenic multifactorial disorders

There is an unmet medical need for the therapy of neurodegenerative disorders; for example, autoimmune, Parkinson's or Alzheimer's disease (AD) and ischemic damage. In such instances, the therapeutic gene might encode a neurotrophic molecule or neuronal survival (antiapoptotic agent), a cytokine, a neurotransmitter or a receptor [13]. Here we describe the current ongoing clinical trials in such polygenic multifactorial neurological disorders. In case of all strategies described below, viral gene transfer is applied for establishing a stable gene reservoir.

### ■ AD

In AD the reduced activity of the cholinergic neurons is a well-known feature. NGF has been shown to prevent cholinergic cell death in rats and primates. NGF has been implicated as a trophic agent in the survival and maintenance of basal forebrain cholinergic neurons [44]. A Phase I AD clinical trial of *ex vivo* delivery of the *NGF* gene expressed in a retroviral vector was completed in 2005 [45]. In 2004 Ceregene started a Phase I clinical study to assess the safety, tolerability and biologic activity of *in vivo* AAV-mediated delivery of CERE-110 (AAV containing the cDNA of *NGF*). All subjects received bilateral, stereotactic injections of CERE-110 injections to target the basal forebrain region of the brain containing the nucleus basalis of Meynert (NBM). All study participants were observed for a 24-month period and then followed annually [226]. Presently CERE-110 is in a Phase II clinical trial in patients with mild to moderate AD. This study will

evaluate safety and efficacy of Ceregene's CERE-110 for the treatment of AD. Approximately 50 people with AD will participate in this study. Half of the study subjects will undergo brain surgery to receive CERE-110, while the other half will undergo a similar surgical intervention without receiving CERE-110 ('sham' or 'mock' surgery) [227].

■ **Parkinson's disease**
There are three different gene therapeutic approaches for treating Parkinson's disease: modulation of neuronal phenotype; neuroprotection; and dopamine level augmentation.

Modulation of neuronal phenotype
The aim is the restoration of dopamin levels in the basal ganglia to prevent or modify the secondary changes due to the dopamin deficiency. In this way, the activity of the neurons of subthalamic nucleus (STN; which gives rise to signals that cause bradykinesia and tremor in Parkinson's disease) can be normalized. In a placebo-controlled, randomized trial, a compound named AAV2-GAD was tested [228]. During this study, the rAAV2 virus was introduced into the neurons of the subthalamic nuclei stereotactically through a small canula in 55 patients. The virus contained the *GAD* gene which prompted the cells to produce GAD, which promotes the production of GABA, an inhibitor that attenuates excessive neural activity associated with Parkinson's disease. Significant improvements in Unified Parkinson's Disease Rating Scale (UPDRS) score were noted in the experimental group compared with the sham group in the off-medication motor scores. UPDRS score at 6 months for the AAV2-GAD group decreased by 8.1 points ($p < 0.0001$) and by 4.7 points in the sham group ($p = 0.003$). The AAV2-GAD group showed a significantly greater improvement from baseline in UPDRS scores compared with the sham group at 6 month. Only mild or moderate adverse events were detected, all likely related to surgery and resolved subsequently. The most common were headache and nausea [46]. A long-term follow-up study is currently ongoing. Patients will be followed once a year for 5 years. The study will monitor and evaluate the long-term efficacy and safety of AAV2-GAD.

Neuroprotection
A Phase IIb study conducted by Ceregen is evaluating the safety and potential benefits of CERE-120 for Parkinson's disease [229]. CERE-120 is an experimental drug that has been safely tested in more than 60 people with Parkinson's disease. An AAV2 vector was selected to transfer the neurturin directly into brain cells most affected by Parkinson's disease. Neurturin is a neurotrophic factor that has been demonstrated in laboratory experiments to restore damaged dopamine cells and protect them from further degeneration [47]. CERE-120 is administered via bilateral stereotactic injections targeting the putaminal region of the brain. The study was completed in 2011 with 51 patients enrolled [230]. In this completed Phase IIb study, a fourfold higher CERE-120 dose was injected into the putamen, as compared with prior studies to help enssure that all the degenerating neurons are exposed to sufficient neurotrophic factor, stimulating a more robust neurotrophic response, which in turn, should further enhance and accelerate neuronal repair and clinical benefit. Another Phase IIb safety and efficacy study is currently ongoing, but not yet recruiting participants [231].

Dopamine level augmentation
Injecting an adenoviral vector containing DNA encoding the *AADC* gene into the striatum of patients suffering from Parkinson's disease may increase local conversion of exogenous levadopa to dopamine. Following gene transfer, the degree of converison can be controlled by changing the levadopa dosage. Phase I studies after 6-month evaluation revealed modest improvement in UPDRS scores, but three out of ten subjects in a US trial suffered hemorrhage along the trajectory of the injecting catheter; Phase II trials have not yet begun [48,49].

Another approach utilizes intrastriatal injection of a multicistronic lentivirus (EIAV-SIN) encoding tyrosine hydroxylase, aromatic amino acid decarboxylase and GTP cyclohydrolase – so called ProSavin [50]. The strategy is also based on the augmentation of dopamine levels, similarly to the aforementioned compound. A nonpulsatile production of dopamine might reduce the incidence of dyskinesias. Oxford Biomedica conducted a Phase I/II trial. The initial phase was a dose-escalation study to evaluate two different dosages (one and two-times) in a small cohort of patients. Oxford Biomedica announced that both doses were well tolerated, improved motor function and quality of life [50]. They initiated a two-stage Phase I/II study [232], in which the first stage is an open-label dose escalation to evaluate up to three dose levels of ProSavin in cohorts of three patients each. In the second stage of the trial, a further 12 patients will be recruited to confirm efficacy of the optimal dose in the randomized phase of the study.

■ **Pain**
NP2, a replication-defective HSV-based vector, which expresses human preproenkephalin (*PENK*) was used

in a Phase I trial. The uptake of *PENK* genes by neurons of the targeted dorsal root ganglia leads to production of preproenkephalin – an opioid precursor protein – which is then cleaved to produce endogenous opioids that inhibit pain signaling in the spinal cord. The ten participants received intradermal injections corresponding to the distribution of the pain. Subjects receiving the low dose of NP2 reported no substantial change in pain, while subjects in the middle- and high-dose cohorts reported pain relief as assessed by numeric rating scale and short-form McGill pain questionnaire [51]. Presently, a Phase II trial is ongoing. The purpose of this study is to examine the impact of intradermal delivery of NP2 on pain scores and pain-medication usage in subjects with intractable pain due to malignant disease. A secondary purpose is to investigate safety and secondary efficacy end points [233].

## Gene therapy for the treatment of malignant neoplasm

### ■ Malignant glioma

The aim of these gene therapeutical treatments is to destroy tumor cells and/or increase the tumor cells' sensitivity to anticancer drugs [52]. Here we discuss some strategies targeting malignant gliomas.

### ■ Gene transfer with oncolytic replicating viruses

Toca 511 utilizes a retroviral replicating vector (RRV) that only infects mitotic cells. The virus expresses the prodrug-activator cytosine deaminase (*CD*), a gene that catalyzes the intracellular conversion of the antifungal drug 5-FC to a cytotoxic drug 5-FU. A Phase I/II study run by Tocagen, Inc. of a retroviral replicating vector to treat subjects with recurrent malignant glioma is currently recruiting participants [234]. This is a multicenter, open-label trial to investigate the safety and tolerability of increasing, single doses of Toca 511, administered transcranially to subjects with recurrent high-grade glioma (HGG) who have undergone surgery, radiation therapy and chemotherapy with temozolomide. Approximately 3–4 weeks following injection of the RRV, 6-day courses of treatment with oral 5-FC will commence and will be repeated monthly, up to six cycles.

Another study by Tocagen, Inc, is also recruiting patients for the evaluation of the retroviral replicating vector to treat patients undergoing surgery for a recurrent malignant brain tumor [235]. This is a multicenter study evaluating the safety and tolerability of Toca 511 injected into the resection cavity of patients with recurrent or progressive grade III or IV gliomas. Approximately 7 weeks after the application of Toca 511 the patients will be treated with 5-FC, an oral antifungal antibiotic for an 8-day course. These 8-day courses of 5-FC will be repeated a total of three-times during the 6-month study. MRI scans will be performed approximately every 2 months. Three subjects will be evaluated at up to four-dose levels of Toca 511. The dose of Toca 511 a patient receives will depend upon the number of previous study participants and how well they have tolerated the study drugs. All patients enrolled in this study will be encouraged to participate in a continuation protocol that enables additional 5-FC administration and the collection of long-term safety and efficacy data.

### ■ Transfer of chimeric antigen receptors to CMV-specific cytotoxic T lymphocytes

Baylor College of Medicine researchers at Texas Children's Cancer Center and their collaborators at The Methodist Hospital have launched two Phase I clinical studies using immunotherapy to treat glioblastoma multiforme. In a Phase I study autologous CMV-specific cytotoxic T-lymphocytes (CTL) were genetically modified to express chimeric antigen receptors (CAR) targeting the HER2 molecule in patients with HER2-positive glioblastoma multiforme. In the currently recruiting Phase I/II trial patients will be investigated for the evaluation of safety and persistence of escalating doses of autologous CMV-specific cytotoxic CTL CAR targeting the HER2 molecule (FRP5.CD28.CAR) in patients with HER2-positive glioblastoma multiforme [236].

### ■ AON therapy

Trabedersen (AP 12009) is a TGF-β2 specific phosphorothioate that has been successfully tested in Phase I/II studies in patients with recurrent or refractory HGG. It was designed for the specific inhibition of TGF-β2 synthesis. TGF-β2 is overexpressed in more than 90% of HGG [53]. Its level is closely related to tumor progression. Inhibition of TGF-β2 in tumor tissue leads to reversal of tumor-induced immune suppression inhibition of tumor growth and invasion. In patients with HGG, intratumoral treatment with trabedersen is currently evaluated in a pivotal, randomized and active-controlled Phase III study [54]. Additionally, a multinational dose-finding Phase IIb study for the evaluation of efficacy and safety of two doses of AP 12009 compared with standard chemotherapy (temozolomide or procarbazine, lomustine and vincristine combination chemotherapy) in adult patients with confirmed recurrent HGG is currently ongoing [237].

## Conclusion

Based on the results of the recent clinical trials it is clear that, while the enormous advances and

| Executive summary |
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**General aspects of gene therapy**

- Gene therapies are can provide treatment for genetic diseases, establish stable gene reservoirs as sources of therapeutic proteins in multifactorial diseases, destroy malignant cells in neoplasms and produce DNA-based vaccines.
- The DNA-based therapies comprise gene replacement (GR), gene transfer (to upregulate therapeutic protein) and gene editing (to correct the defected gene).
- The RNA-modulating therapies include exon skipping by antisense oligonucleotides, mutant RNA removal, inactivation of the mutant mRNA by RNAi and destruction of the mutant mRNA by ribozymes.

**Gene therapy of genetic disorders**

- GR entails the introduction of functionally adequate alleles into tissues in which deleterious effects of the gene defect is maximal. For optimal GR, the determination of the most appropriate transferable gene, promoter, vector and route of administration is crucial. Clinical trials using GR in several disorders are currently ongoing.
- RNA-modulating therapeutics provide powerful tools for targeted modulation of gene expression by the use of antisense oligonucleotides or siRNA.
- Exon skipping by antisense oligonucleotides represents a promising therapeutic tool for many disorders, in particular for Duchenne muscular dystrophy. The most notable example is exon 51 skipping (present in 13% of all patients). Two Phase I clinical trials were conducted by a Prosensa with GalaxoSmithKlein and AVI-Biopharm using this strategy. In both studies the dystrophin expression increased in a dose-dependent manner. Further Phase I, II and III trials started in 2011.

**Inactivation of the mutant mRNA by RNA**

- In a Phase I trial, ALN-TTR01 is administered intravenously to patients with transthyretrin-mediated familiar amyloid polyneuropathy to block pathogenic accumulations of mutant *TTR* in peripheral tissues.

**Translational manipulation**

- When a primary stop codon due to a nonsense mutation leads to a truncated and unstable protein, corrupting the ability of the ribosomes to recognize stop codons could be beneficial. This approach has been investigated for use in patients with nonsense mutation Duchenne and Becker muscular dystrophy and cystic fibrosis.

**Gene therapy for complex, polygenic multifactorial disorders**

- CERE-110 (AAV-mediated delivery of nerve growth factor) is in a Phase II trial in patients with mild to moderate Alzheimer's disease. There are three gene therapy approaches for treating Parkinson's disease: modulation of neuronal phenotype, neuroptrotection and dopamine level augmentation.
- Restoration of the dopamin level in the basal ganglia to prevent or modify the secondary changes due to the dopamin deficiency. *GAD* gene containing the rAAV2 virus was introduced to the subthalamic nuclei stereotactically through a small cannula in 55 patients. *GAD* promotes the production of GABA, an inhibitor that ameliorates excessive neural activity associated with Parkinson's disease. Significant improvements in Unified Parkinson's Disease Rating Scale score were noted in the experimental group compared with the sham group in the off-medication motor scores.
- Bilateral stereotactic injections of CERE-120 (neurturin, a neurotrophic factor that restores damaged dopamine cells and protects them from further degeneration) targeting the putaminal region of the brain in a Phase IIb study was completed in 2011.
- Injecting an adenoviral vector containing DNA encoding the *AADC* gene into the striatum of patients suffering from Parkinson's disease may increase local conversion of exogenous levadopa to dopamine. Phase I studies after 6-month evaluation revealed modest improvement in Unified Parkinson's Disease Rating Scale scores, but three out of ten subjects in the US trial suffered hemorrhage along the trajectory of the injecting catheter.
- Intrastriatal injection of a multicistronic lentivirus (EIAV-SIN) encoding tyrosine hydroxylase, aromatic amino acid decarboxylase and GTP cyclohydrolase – so called ProSavin – results in the augmentation of dopmaine levels. Oxford Biomedica conducted a Phase I/II dose escalation trial. Both doses were well tolerated and improved motor function and quality of life.

**Gene therapy for the treatment of malignant neoplasm**

- In malignant glioma, the aim of gene therapy is to destroy tumor cells and/or increase the tumor cells' sensitivity to anticancer drugs. These strategies comprise gene transfer with oncolytic replicating viruses or transfer of chimeric antigen receptors to cytomegalovirus-specific cytotoxic T lymphocytes.
- In a Phase I study autologous cytomegalovirus-specific cytotoxic T lymphocytes were genetically modified to express chimeric antigen receptors targeting the HER2 molecule in patients with HER2-positive glioblastoma multiforme.
- Another approach is antisense oligonucleotide therapy. Trabedersen is a TGF-b 2 specific phosphorothioate, that has been successfully tested in Phase I/II studies in patients with recurrent or refractory high-grade glioma. It was designed for the specific inhibition of TGF-β2 synthesis, the level of which is closely related to tumor progression. Inhibition of TGF-β2 in tumor tissue leads to reversal of tumor-induced immune suppression, inhibition of tumor growth and invasion. In patients with high-grade glioma, intratumoral treatment with trabedersen is currently evaluated in a pivotal, randomized and active-controlled Phase III study.

achievements in molecular science helped a great deal with the diagnosis and prevention of genetic CNS and muscle diseases, the etiology of many diseases has not yet been fully elucidated. The therapeutic application of genetic approaches in the routine clinical practice is still anticipated. In fact, at present, only a handful of gene therapies targeting CNS and muscle diseases are being tested in clinical trials. There are still no approved gene therapy products in neurological indication, but numerous experimental and clinical research activities are ongoing in this field. The development of gene therapies has substantial risk both for the sponsor and for the patient. In many of the current clinical trials genetic treatments operate with viral vectors, which may represent a safety risk. The gene transfer can easily induce several processes beyond control, such as tumor progression, autoimmune reactions and further yet-unknown processes. It can be concluded that, although gene therapy is still in the experimental stage and confirmatory results are pending, it holds great promise to substantially impact neurological therapy in the future.

## Financial & competing interests disclosure

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