## ORIGINAL ARTICLES

# Readthrough strategies for stop codons in Duchenne muscular dystrophy

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**Duchenne muscular dystrophy is due to mutations of the dystrophin gene. These are large deletions or duplications in 80% of cases, while premature stop codons (nonsense point mutations) account for 7% of cases. This subgroup of patients may take advantage of the properties of the antibiotic gentamicin to suppress stop codons (readthrough). The efficiency of the readthrough varies inversely to the efficiency of a stop codon and is also affected by the different components of the drug. Following gentamicin treatment of mdx mice, dystrophin was re-expressed up to 20% of normal level, albeit with variability among animals. Human trials with gentamicin have so far obtained doubtful results. PTC124 belongs to a new class of small molecules that mimics at lower concentrations the readthrough activity of gentamicin. The administration of PTC124 resulted in the production of full-length and functionally active dystrophin both in vitro and in mdx mice. A Phase II clinical trial is now in course and will be terminated at the end of 2006.**

**Key words:** Dystrophin, stop codons, nonsense mutations.

## Dystrophin mutations

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are allelic disorders due to mutations of the dystrophin gene (1). This gene spanning 2.4 Mb at Xp21, is the longest of the human genome, and requires 16 hours to be transcribed (2). The main muscular mRNA, composed of 79 exons, encodes a 427kDa-dystrophin. It consists of four distinct domains: N-terminal actin-binding, a larger central rod-like domain, a cysteine-rich domain and the C-terminal domain that binds to the glycoprotein complex (3,4). In general, mutations_causing absence of dystrophin are associated with DMD, while dystrophin reduction and/or rearrangement is found in BMD. In DMD that is an X-linked lethal disease, one-third of all mutated alleles per genera-

tion are removed because they belong to severely affected males that rarely have children. Following Haldane's rule (5), the rate of appearance of mutated alleles must equal their removal: thus, the frequency of new dystrophin gene mutations is particularly high (up to $10^{-4}$) and the mutation spectrum is extremely heterogeneous. Clearly, methods of prenatal diagnosis, based on relatives of known cases, cannot reduce the future incidence of DMD beyond this limit.

In the majority of patients, the gene is broken by deletions that span up to hundreds of thousands of DNA nucleotides, encompassing one or more exons.

Gross deletions are easily diagnosed worldwide by multiplex polymerase chain reaction (PCR), but, in about one-third of cases, deletions are absent. In the last few years, a number of these missing mutations have been detected: disease may be due, in order of frequency, to: 1) exon and exon-flanking small mutations; 2) duplications; 3) deep intronic mutations; 4) translocations; 5) insertion of repetitive sequences. It is difficult to accurately estimate the frequency of the different mutation types, since the published figures (6) are biased by the low sensitivity of some mutation discovery methods and by the focusing of efforts on selected regions of the gene. According to our data, in DMD, small nucleotide insertion and/or deletions account for 7%, splice site mutations 2%, nonsense mutations 7%. In contrast, in BMD patients, most small mutations affect the splicing. As a general rule, the effects on the phenotype, in the case of a nonsense mutation, depend on the position in the cDNA, since, in theory, a truncated protein could be produced. However, in the case of dystrophin, the preservation of the domains encoded by the 3' exons is crucial for the stability of the complex with the

associated proteins at the sarcolemma. Thus, most nonsense mutations that occur before exon 70 are associated with DMD. There is only one exception: nonsense mutations that occur in exons that may be alternatively spliced with preservation of the reading frame. In these cases, the stop codon is partially spliced off and a small amount of dystrophin is produced, resulting in BMD or intermediate phenotypes.

## Treatment options

Although the molecular bases of DMD were clarified twenty years ago, the development of successful therapy has, nonetheless, remained a discouraging challenge. Even if life expectancy has been much extended by pharmacological treatments and assisted ventilation, DMD remains a fatal condition. All the options for therapeutic strategies can be classified into two large groups: i) *primary,* which aim to regain dystrophin; ii) *secondary*, i.e., all the efforts aim to modify disease progression without restoring dystrophin (7,8).

Various secondary approaches have been explored, such as new pharmacological and cell-based therapies, cytokine and genetic therapies, that are targeted to specific features of dystrophic DMD muscle disease. While these approaches aim to produce immediate benefits for the patients, the primary approach is by far more challenging, even if much more problematic. The large size of the dystrophin gene has required the development and use of vectors containing the best-working mini- or micro-dystrophin cDNA. Some researchers have attempted to target the muscle stem cell population (satellite cells) *in situ* through *in vivo* administration of a pseudotyped lentiviral vector encoding the mini-dystrophin. These studies showed that progenitor cells can be genetically engineered and subsequently proliferate into terminally differentiated tissue providing functional correction of the muscle restoring dystrophin expression (9). Chamberlain et al. obtained an efficient systemic delivery by high-dosage of pseudotyped adeno-associated viruses (AAV). Micro-dystrophin was delivered to all muscles, both in young and aged utrophin/dystrophin double K.O. animals, with manifest therapeutic effects (10-12). However, equivalent AAV dosage are not yet realistic/feasible in humans.

Alternatively, several other attempts can be made to recover the dystrophin expression when the majority of the gene is maintained. This can be obtained: i) by forcing the splicing machinery to induce the specific skipping of mutated exons or to produce out-of-frame to in-frame conversions; ii) by forcing translation machinery to overcome stop codons (readthrough) (13).

## Aminoglycosides

Readthrough strategies take advantage of the known properties of a category of drugs, the antibiotic aminoglycosides that can suppress stop codons.

As antibiotics, aminoglycosides cope with bacterial infections by interfering with the prokaryotic translation. Although these drugs are specifically active against bacterial ribosomes, they also have a minor effect on cytoplasmic ribosomes in eukaryotic cells by disturbing the translation machinery (14). The sensitivity of human ribosomes to some aminoglycosides has been viewed as an unwanted side-effect. In eukaryotes, stop codons are decoded on the ribosome. Several members of this antibiotic family (gentamicin, tobramycin, amikacin, hygromycin, etc.) can suppress the accurate identification of translation termination codons in cultured eukaryotic cells, as a particular usage of their general ability to decrease the fidelity of translation (15). In principle, aminoglycosides induce miscoding by mimicking the conformational change in rRNA induced by a correct codon-anticodon pair, thereby compromising the integrity of codon-anticodon proofreading during translation (Fig. 1). As a general rule, glutamine is inserted at nonsense UAG or UAA readthrough sites, whereas UGA sites miscode to tryptophan (16). The aminoglycosides may also influence the ability of release factors, such as RF1 and RF2, to stabilize the nascent protein strand in the ribosome for further elongation (17). In brief, by allowing readthrough past the premature stop codon, a full functional protein should be produced. Aminoglycosides can be quite effective in inducing readthrough (18-20). The efficiency of the readthrough varies inversely to the efficiency of a stop codon, in humans the ranking order for aminoglycoside readthrough efficiency generally being UGA>UAA>UAG (21). This ranking varies from one species to another, and is also influenced by context within a stop codon appears, especially the flanking region of +4 nucleotides (22, 23). The *in vitro* treatment of eight premature stop codon mutations, identified in DMD/BMD patients, shows that one stop codon mutation is suppressed significantly better (up to 10% readthrough) than the
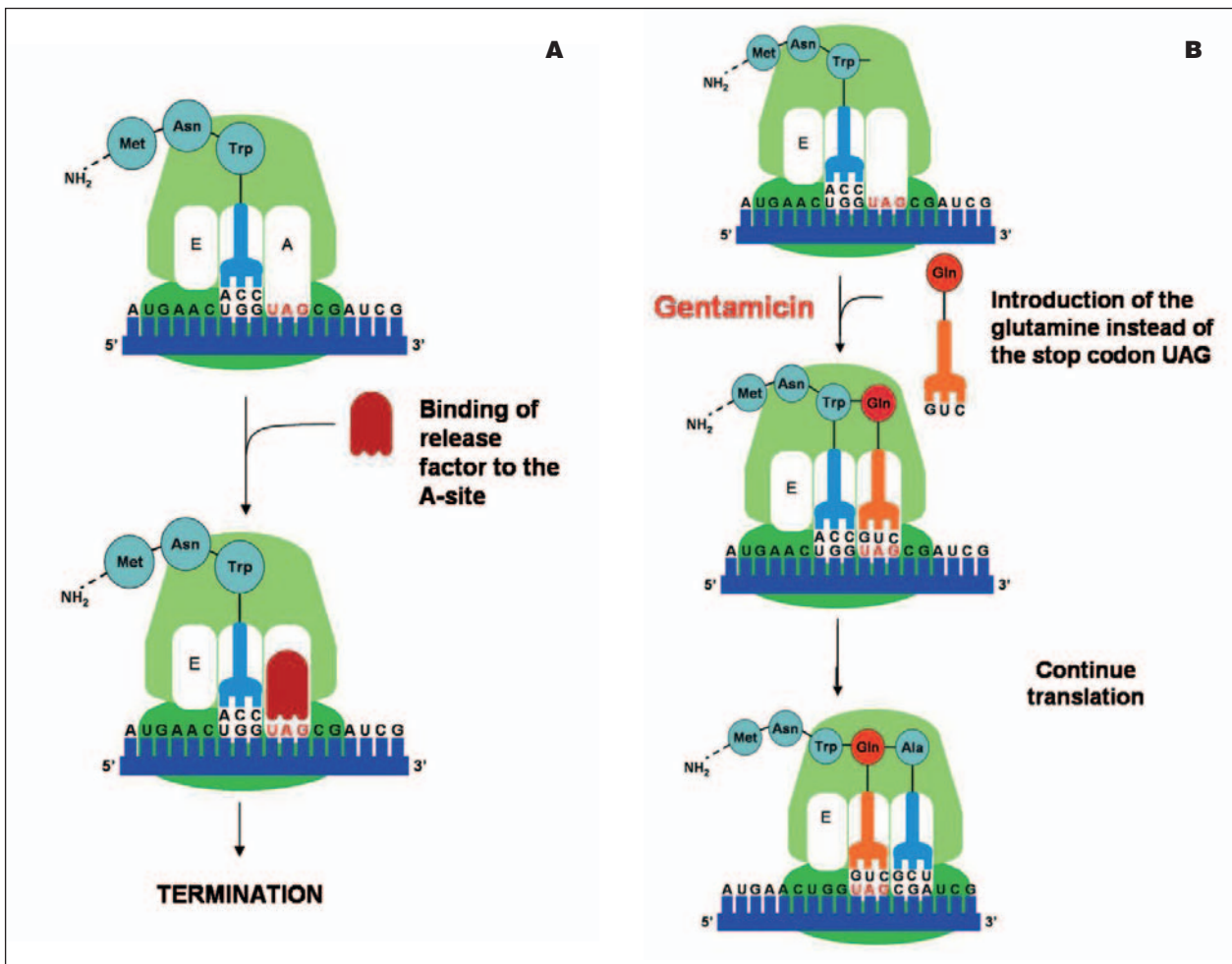
**Figure 1.** Transcription is stopped due to presence of premature stop codon (UAG) in mRNA.
Presence of antibiotic gentamicin forces translation machinery causing readthrough of stop codon introducing a glutamine.
*Modified from Molecular Biology of the Cell 4th edition, Alberts et al. – Garland Publishing*

others; five show lower, but statistically significant, suppression (< 2% readthrough), and two appear refractory to aminoglycoside treatment (24).

The induction of random errors, in dystrophin, does not complicate this approach, since dystrophin seems to be tolerant to the majority of single amino acid substitutions that are usually found in healthy individuals (6). However, the loss of fidelity may lead, in theory, to global mistranslation, since any other mRNA could be misread.

A potential complication, for the treatment of DMD patients, is the toxicity of aminoglycoside antibiotics during long-term/-life treatments that may result in nephrotoxicity (25) and ototoxicity (26).

The commonly used gentamicin sulphate (Gentamicin C) is a mixture of three major components designated as C1, C1a and C2 which have different patterns of methylation at the 6' position of ring I (Fig. 2). *In vitro* studies showed that the three components of gentamicin C bind to the A-site of the 30S ribosomal subunit at the same binding site, but with different affinities; gentamicin C1a having slightly higher affinity than C2, whereas C1 binds with the lowest affinity (20-50-fold weaker) (27).

According to the Sigma-Aldrich product information, the ratio of the three major components by High power liquid chromatography (HPLC) analysis is: C1: < 45%, C1a: < 35% and C2: < 30%. It cannot be excluded that different batch preparations may differ in the component proportions, resulting in stronger or weaker therapeutic effects. It is, therefore, advisable to perform a HPLC analysis of the gentamicin batch used for delivery.
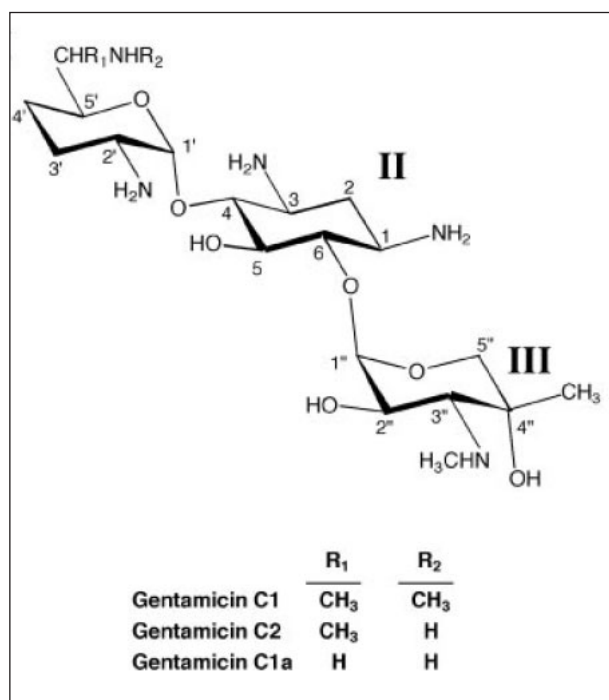
**Figure 2**. Gentamicin C is a mixture of three major components: gentamicin C1, C1a and C2 that have different pattern of methylation at 6' position of ring 1.

## Aminoglycosides in mice

The first evidence of a possible therapeutic use of this side-effect of aminoglycosides came from experiments on HeLa cell lines carrying two common, disease-associated mutations in the cystic fibrosis gene (CFTR), a stop codon in place of glycine residue 542 (G542X) and arginine residue 553 (R553X). The termination was suppressed by treatment with low doses of the aminoglycoside G-418 or gentamicin in a dose-dependent manner (28). G-418 and gentamicin were also capable of restoring CFTR expression in a bronchial epithelial cell line carrying the CFTR W1282X premature stop mutation (29). Following these promising observations, Sweeney et al. treated the *mdx* mouse (18). This is the best known animal model for dystrophin deficiency and carries a nonsense mutation in exon 23 of the dystrophin gene (glutamine mutated into a stop codon UAA) (30). They observed that gentamicin could lead to increased expression of dystrophin and restoration of the dystrophin complex at the sarcolemma together with improved resistance to lengthening contractions. Dystrophin levels were up to 20% of normal mouse muscle, suggesting that misreading of RNA could be sufficient to protect the muscles from contraction-induced damage. In humans, this level of expression is found in BMD rather than in DMD patients. In the study, not all animals responded to gentamicin, possibly owing to different rates of antibiotic metabolism. In addition, the animals that responded did so over a very narrow concentration range of gentamicin.

Method of administration also influenced the outcome. Loufrani et al. (31) showed that gentamicin treatment (34 mg/kg per day for 14 days) of mdx mice produced 40% of dystrophin recovery. The treatment restored endothelial response to flow that appears markedly reduced in *mdx* mice. Immunoblot experiments showed the recovery of a full-length protein that was detected by antibodies directed against the C terminus, the N terminus, and the mid rod domain of the protein.

In contrast, Dunant et al. were unable to replicate the effects of gentamicin in mdx mice (32).

Arakawa et al. reported that negamycin, a dipeptide antibiotic that seems to be less toxic than gentamicin, can be as efficient in restoring dystrophin expression in skeletal and cardiac muscles of the mdx mouse and in cultured mdx myotubes (33).

## Novel products: PNAs and PTC124

An attempt to avoid aminoglycoside toxicity is the use of antisense peptide nucleic acids (PNAs). In vitro studies showed the ability of PNAs to force the readthrough of a stop codon in the reporter gene. The activity was increased 2.5-fold over control levels, similarly gentamicin. These observations are preliminary and cannot solve the problem of administration and delivery of molecules (34).

PTC124 belongs to a new class of small molecules developed by PTC Therapeutics, Inc.(South Plainfield, New York, USA) It is an orally bioavailable drug, no antibiotic, that mimics the activity of aminoglycosides and allows ribosomes to bypass the nonsense mutations in mRNAs and continue the translation process to make full-length and functional protein instead of the truncated one (35).

The PTC124-induced readthrough was assessed in HEK293 cells transfected with a luciferase gene harbouring a premature stop codon at Thr190, replacing the normal ACA with UAA, UAG and UGA. The experiments showed onset of nonsense suppression within 2 hours from drug administration and maximal activity within 20 hours; the loss of activity by 6 hours after drug removal. PTC124 activity was codon- and concentration-dependent. At 20 hours,

maximal readthrough activity over control was 12-fold (UGA), 4-fold (UAG) and 2-fold (UAA). With each codon type, readthrough was discernable at a concentration about 100- to 1000-fold less than gentamicin (about 0.1 μM - 1.0 μM instead of 100 μM for gentamicin) (36). The FDA has granted fast track designation and orphan drug designation to PTC124 for the treatment of both cystic fibrosis and DMD caused by nonsense mutations. The European Medicines Agency (EMEA) has granted orphan drug status to PTC124 for the treatment both of cystic fibrosis and DMD.

## PTC in mice

The administration of PTC124 resulted in the production of full-length and functionally active dystrophin both in in vitro and in mdx mice.

Different groups of animals were treated with two different doses of the drug (0.9 mg/mL and 1.8 mg/mL) for 8 weeks. Plasma concentrations of PTC124 were similar at both dose levels; dystrophin was restored similarly at both dose levels; it was absent in untreated mdx mice and normally expressed in wild type mice (Fig. 3). Levels of serum creatine phosphokinase kinase (CPK) were slightly reduced with the treatment. Moreover, there was no evidence that PTC124 would induce changes in ribosomal readthrough of normal stop codon based on western blot experiments assaying protein elongation in different tissues (37).
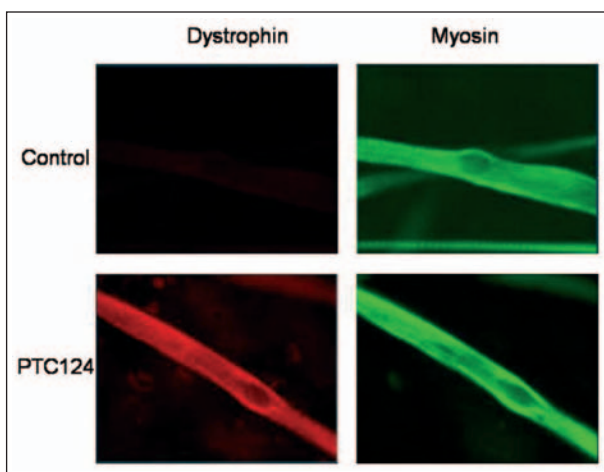


**Figure 3.** Immunofluorescence images of myotubes from mdx mice stained for dystrophin (left panels; red) and myosin (right panels; green). Oral administration of PTC124 results in production of dystrophin at skeletal muscle, as measured by immunofluorescence.
*From PTC Therapeutics, Reproduced with permission.*

## Human trials with gentamicin

Success has largely been limited to tissue culture and *in vitro* experiments. Gentamicin has already been in use for many years and its side effects are well-known. For its simplicity and low cost, it deserves scrupulous attention in order to evaluate whether some patients have true improvements. Negative trials in humans were reported that failed to observe an increase in dystrophin expression. Wagner et al. treated two BMD and two DMD patients, with Q625X (UAAG), S757X (UGAG), Q2198X (UAGC) and W3293X (UGAC) stop codon sequences (38). They administered gentamicin once daily with intravenous gentamicin at 7.5 mg/kg/day for 2 weeks. No ototoxicity or nephrotoxicity were detected. Gentamicin was unsuccessful in producing detectable full-length dystrophin in post-treatment muscle biopsies. In all these patients, serum CPK levels were reduced, but this was not due to treatment, but to less activity. On the other hand, Politano et al. (39), reported the treatment of four DMD patients, three with UGAC and one with UAAA stop codons, still ambulant, with two 6-day cycles of 7.5 mg/kg/day gentamicin, at an interval of 7 weeks. Three out of four patients, who had the most permissive UGAC as stop codon, showed some positive results at the final muscle biopsy. In one patient, there was an evident re-expression of dystrophin observed by both immuno-histochemistry and Western blot; in two other patients, dystrophin positive fibres were seen by the antibody to the rod domain, while the fourth patient, with UAAA as stop codon, showed no expression of dystrophin at all. The Authors concluded that gentamicin is able to recover dystrophin expression in a subset of Duchenne patients with nonsense mutations. The most striking result was observed with the DMD patient with a stop codon in exon 29 (Arg1314X). Gentamicin restored the dystrophin expression and improved the phenotype. This same nonsense mutation, found three other times, however, has been associated with BMD, intermediate D/BMD and X-linked cardiomyopathy phenotypes (6). This variability is due to the fact that exon 29 may be normally spliced out in a fraction of mRNA. There is the possibility that, with this particular mutation, the resulting phenotype is borderline and that dystrophin expression could be more easily restored.

A Japanese group, in order to develop a system for identifying candidate patients, isolated fibroblasts from nine patients with DMD nonsense mutations and induced myogenic differentiation. The cell lines were exposed to gentamicin and the dystrophin

expression in treated myotubes was monitored by in vitro staining and immunoblot analysis. The authors showed that gentamicin was able to induce dystrophin expression by the readthrough of the nonsense mutation UGA. The nonsense UAA and UAG did not occur as well (40). Bidou et al. (41) developed a sensitive dual reporter assay to test the suppression level induced by gentamicin on premature stop codons in dystrophin gene. They showed that the effect of gentamicin on readthrough is similar in cultured cells and *in vivo* in murine skeletal muscle and thus preliminary assays in cell culture provide valuable information concerning the potential efficiency of pharmacological treatments. They found that only a minority of premature stop codons found in patients show a significant level of readthrough, and would thus be amenable to this pharmacological treatment.

## Human trials with PTC124

PTC Therapeutics carefully analyzed the effect of the drug in the tissue culture system and in animal models (pre-clinical study) following two Phase I clinical trials involving a total of 61 healthy volunteers.

PTC124 was administered orally as a liquid suspension.

It was determined that 100 mg/Kg is the maximum tolerated dose, based on the observation of headaches, dizziness and mild gastrointestinal events, such as nausea, vomiting and diarrhoea, at 150 mg/Kg and 200 mg/Kg doses. PTC124 was palatable, with no odour or taste. There were no clinically significant adverse events reported, at any dose tested, although modest increases in liver enzymes were observed in some subjects. PTC124 achieved and maintained the plasma concentrations that may have a therapeutic effect.

The improper readthrough of normal stop codons was assessed by observing whether the participants in the trial produced larger forms of specified proteins.

By the end of 2005, PTC initiated the phase II clinical trials (42). This means recruiting patients. The eligibility criteria for enrollment are the absence of dystrophin, on a muscle biopsy, and the presence of a nonsense mutation, in the dystrophin gene, confirmed by the University of Utah (43). Furthermore, patients must have the ability to walk and be at least 5 years old. Details on the phase II clinical trial, eligibility of patients and administration of the drug are available online (www.clinicaltrials.gov 35,42). The expected date for completion of the project is December 2006. PTC intend to conduct a phase III trial to evaluate long-term safety of PTC124 and to support registration of the drug regulatory authorities.

## Conclusions

Pharmacological readthrough strategy may be a ready-to-go alternative to recover a part of dystrophin at the sarcolemma. A modest amount of dys-

**Table 1.** Frequency of different codons found in 534 DMD patients with nonsense mutations reported (6). Altogether nonsense mutations represent about 7% of DMD causes

| CODON and +4 base | No. cases | Nonsense mutations % | Total mutations % | Expected readthrough efficacy |
|---|---|---|---|---|
| UAAA | 54 | 10.1 | 0.71 | * |
| UAAC | 23 | 4.3 | 0.30 | * |
| UAAG | 48 | 9.0 | 0.63 | * |
| UAAU | 10 | 1.9 | 0.13 | * |
| *all UAA* | *135* | *25.3* | *1.77* | |
| UAGA | 54 | 10.1 | 0.71 | ** |
| UAGC | 48 | 9.0 | 0.63 | ** |
| UAGG | 42 | 7.9 | 0.55 | ** |
| UAGU | 36 | 6.7 | 0.47 | ** |
| *all UAG* | *180* | *33.7* | *2.36* | |
| UGAA | 88 | 16.5 | 1.15 | *** |
| UGAC | 42 | 7.9 | 0.55 | ***** |
| UGAG | 70 | 13.1 | 0.92 | *** |
| UGAU | 19 | 3.6 | 0.25 | **** |
| *all UGA* | *219* | *41.0* | *2.87* | |

trophin (10%) could slow down the dystrophic process and improve the quality and duration of life. Clinical trials, however, require several specific conditions for the recruitment of DMD patients. First, there must be one single stop codon. This excludes all frame-shift mutations from this treatment (deletions, duplications, small insertions/deletions, splice mutations, etc.) that are the vast majority (93%) of the DMD defects. Second, the nonsense mutation should not be associated with a significant decay of the dystrophin mRNA level (44). Third, the nonsense mutation should be UGA and occur at certain positions along the sequence in a favourable DNA sequence context.

About 0.8% of all DMD patients should have UGAC or UGAU and are eligible for this treatment (Table 1) while an additional 2.0% partially meet the requirements and should be included in a separate group. Considering the incidence of DMD, at least 200 DMD patients in Europe should have the most favourable stop codon contexts and an additional 500 may also be treated. Unfortunately, it is very difficult to recruit patients that are amenable to this therapy, because, in most countries, genetic tests are limited to discover gross deletions. They remain incomplete since it is time-consuming and expensive to identify point mutations in this complex gene. Muscular dystrophy associations (i.e., Parent Project, Telethon-UILDM), however, are supporting projects that aim to perform free-of-charge sequence analysis in DMD patients.

## Acknowledgements

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43. See the website http://www.clinicaltrials.gov the project ID is PTC124-GD-004-DMD

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