

A proposed extension of Ataluren indications (with future deserved studies) in patients with Duchenne muscular dystrophy (DMD) caused by frameshift mutations of dystrophin gene associated with abnormal premature termination codons (PTCs) at distance from the site of that given frameshift mutation

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0. Abstract (with main abbreviations used in this paper)

This paper proposes an extension of Ataluren indications (with future deserved studies) in patients with [Duchenne muscular dystrophy \(DMD\)](#) caused by [frameshift mutations](#) of [dystrophin \(dys\)](#) gene (dys-gene; aka “DMD gene”) associated with abnormal premature stop codons at distance from the site of that given frameshift mutation (which mutation may affect the dys-gene starting from exons with high index more close to 79 than to 1): some strong arguments are presented in favor of this new extension proposal; a redefinition of nonsense mutation in both [stricto sensu](#) and [lato sensu](#) are also presented (emphasizing that frameshift mutations [FSMs] and nonsense mutations [NSMs] [in lato sensu] may come in association).

1. A short introduction on Duchenne muscular Dystrophy (DMD)

The etiology of DMD [1, 2, 3, 4]. [Duchenne Muscular Dystrophy \(DMD\)](#) [5,6] is the most common type of [muscular dystrophy](#) and has an incidence of ~1/3600 born male infants. DMD is a severe [X-linked recessive](#) muscular dystrophy caused by a mutation (inherited from a person's parent in ~2/3 of DMD cases and non-inherited de novo mutation in ~1/3 of DMD cases) in the gene encoding [dystrophin \(dys\)](#) (**dys-gene**) (which is [the largest known human gene](#) composed from ~2.5 million bases [[~0.08% of the total human DNA/genome](#)] and located on the short [p] arm of [X chromosome](#), at locus 21 [Xp21]), which dys is a [cytoplasmic](#) protein and an essential component of a protein complex (with many subunits) (aka [costamere](#) or [the dystrophin-associated protein complex \(DAPC\)](#) [[URL2a](#), [URL2b](#)]) that connects the [myocyte cytoskeleton](#) to the surrounding [basal lamina](#) (an important component of the [basement membrane](#) of the [extracellular matrix](#))

through the muscular cell membrane (aka [sarcolemma](#)): this dys-mediated anchoring of the sarcolemma to BOTH the [basal lamina](#) AND the underlying cytoskeleton (of the myocyte) strongly protects the sarcolemma against the mechanical stress imposed during [muscle contraction](#) and/or [stretch](#): the major functional deficit in the muscles of DMD patients is an increased susceptibility to injury which results in repeated cycles of degeneration-regeneration, important intracellular oxidative stress with secondary ongoing inflammation, and necrosis, with the eventual destruction of muscle [[URL](#)] [[URL](#)].

The pathogenic causal chain of DMD plus the essential consequent clinical elements. Normal skeletal [myocytes](#) contain small amounts of dys but its total/partial absence or abnormal length leads to excess [calcium cations](#) (Ca²⁺) penetrating the sarcolemma and causing excess water to enter into all [mitochondria](#) which then burst, causing [intracellular oxidative stress](#), sarcolemma permanent damage and [myocytes/cardiomyocytes necrosis](#). Progressive [rhabdomyolysis](#) causes muscular fibers to be progressively replaced by adipose and connective tissue ([pseudohypertrophic](#) muscular dystrophy or [muscular pseudohypertrophy](#)). [Muscle weakness](#) associated with progressive [muscle atrophy](#) (with secondary [fatigability](#), frequent falls and progressive difficulty in walking and getting up from lying or sitting position) usually begins around the age of 4 years and worsens rapidly in boys with DMD, so that most of them become unable to walk by the age of 12 years. In advanced stages, DMD patients may have respiratory disorders (due to respiratory muscles damage), swallowing difficulties (with high risk of aspiration pneumonia) etc.

Essential paraclinical elements of DMD patients (laboratory tests and imagistics). Due to rhabdomyolysis (including myocardium cytolysis), DMD patients have extremely high [Creatine kinase \(CK\)](#) and possibly (very) high [CK-MB isomer \(CK-MB\)](#) serum levels: the serum levels of [Aspartate transaminase \(AST\)](#) and [Alanine transaminase \(ALT\)](#) are also very increased. Consequently, [myoglobin \(MG\)](#) (produced by rhabdomyolysis) also attains high concentrations in serum and urine. [Electromyography \(EMG\)](#) distinguishes the weakness caused by destruction of muscle tissue. [Echocardiography](#) may show [dilated cardiomyopathy](#) secondary to [myocardial fibrosis](#) (which can occasionally lead to [congestive heart failure](#) and/or [cardiac arrhythmias](#)). [DNA testing](#) (by both [MLPA](#) and [dys-gene sequencing](#)) demonstrating mutation(s) in one or more of the 79 exons of dys-gene can often make the diagnosis at birth or confirm the diagnosis in most suspected cases.

The differential diagnosis of DMD patients. Among the differential diagnosis of DMD are other genetic/non-genetic muscular dystrophies (MDs), from which the more rare (1.5-6/100 000 male births) (X-linked recessive) [Becker muscular dystrophy \(BMD\)](#) is similar to DMD (regarding etiology and pathogenesis which include less severe mutations of the same dys-gene [thus making BMD a dystrophinopathy too]) but with less affected (“milder”) phenotypes.

Current and perspective treatment of DMD patients. DMD doesn't have a curative treatment, but only a [pathophysiological](#) and symptomatic treatment which may delay the onset of symptoms and increase the quality of life. *Several types of medications* were proved to be relatively useful in the treatment of DMD and its

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complications, such as:

- (1) [steroids](#) such as [prednisolone](#) and [deflazacort](#) (which were demonstrated to slow muscle degeneration and to produce short-term improvements in muscle strength and function up to 2 years, including walking period prolongation according to some reports);
- (2) [β2-agonists](#) such as [salbutamol](#) (which were demonstrated to increase muscle strength, but don't modify disease progression);
- (3) [anticonvulsants](#) (for possible seizures control);
- (4) [ataluren](#) (aka [PTC124](#), with "PTC/PTCs" being an abbreviation for "premature [termination/stop codon/codons](#)"[TC(s)/SC(s)], which TC/SC is a [nucleotide triplet](#) [within a [messenger RNA \[mRNA\]](#) molecular strand] that signals a [translation stop](#) of that TC-containing mRNA into proteins): ataluren orally is indicated for DMD patients that can walk and are more than 5 years old; ataluren makes [ribosomes](#) less sensitive to PTCs, especially for the 'UGA' [stop codon](#) (3 times more effective than on UAG and 6 times more effective than on UAA), by promoting insertion of certain near-cognate [transfer RNA \(tRNA\)](#) at the site of PTCs, with no apparent effects on downstream mRNA transcription, processing, stability nor on the resultant protein); ataluren has a selectivity for the [ribosomal A site](#) (with [tobramycin](#), a compound with a demonstrated high affinity for the same ribosomal A site, being a strong inhibitor of ataluren, probably by competition) and has been selected as the most potent PTC-readthrough promoting drug from over 800,000 compounds screened [[URL](#)]; ataluren was also tested in other (non-DMD) genetic disease caused by NSMs of various genes: [cystic fibrosis](#), [Miyoshi \(distal muscular\) myopathy](#), [Usher syndrome](#), [Batten disease](#), [Alpha-1 Antitrypsin-Deficiency \(A1AD\)](#) [[URL](#)] [[URL](#)];
- (5) [Sildenafil](#) (which was also demonstrated to improve the muscular blood flow in DMD boys) etc.

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There are also several new genetic treatment approaches to DMD patients:

- (1) The [exon-skipping gene therapy \(ESGT\)](#) with [antisense oligonucleotides](#) (oligos/AONs like [eteplirsen](#) or [drisapersen](#)) triggers skipping of an exon (adjacent to the exon affected by mutation) so that to restore the reading frame and production of a (still-truncated but) more functional version of dys. For ESGT to be efficient on medium and long term, AONs must be periodically redelivered into muscles.
- (2) [Stem cell replacement therapy \(SCRT\)](#) was also proposed. SCRT is a therapy using [pericytes](#) (a type of [multipotent stem cells](#) which have the ability to be delivered systemically and uptaken by crossing the [vascular barrier](#), then to fuse and form [myotubes](#)): pericytes are injected arterially, crossing through arterial walls into muscles, where they can differentiate into potentially functional myocytes;
- (3) [CRISPR/Cas9-mediated genome editing](#) (not currently feasible in humans, but potentially feasible in the future) is the most ambitious hope in the treatment of DMD: this is a technique which can precisely remove a targeted mutation of dys-gene, by allowing the DNA repair mechanisms of myocytes to replace that mutant dys-gene with a normal dys-gene.

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Prognosis of DMD patients. Despite all these efforts in

finding new and more efficient treatments, the average life expectancy of DMD patients is ~ 26 years (with a maximum between 30-50 years in rare cases who benefit from excellent care). Most DMD patients become [wheelchair-dependent](#) early in life and the gradual development of [cardiac hypertrophy](#) and/or [restrictive respiratory failure](#) typically results in [premature death](#) between ages of 20-30 years.

2. An introduction on ataluren (aka PTC1124)

The known pharmacological mechanism of ataluren in detail (1). Ataluren promotes readthrough of each of the three RNA premature [stop codons](#) (UAG, UAA and UGA), which may be accidentally produced by a so-called [nonsense mutation](#) carried by some DMD patients. **Note.** [Gentamicin](#) for example, has a similar effect but at much higher intracellular concentrations (implying a relatively high [nephrotoxicity](#) and [ototoxicity](#) at those high doses). More specifically, ataluren produces a 3D conformational change in the mRNA structure, a change which allows the ribosomal subunits to translate (by substitution) a mutation-induced PTC into a single amino acid and to continue translation after the locus/place/position of occurrence of that PTC [[URL1](#), [URL2](#)].

Correlating inversely with established termination efficiencies [[URL1](#), [URL2](#)], readthrough is highest at UGA, followed by UAG and then UAA. With all three RNA stop codons, the minimal concentration of ataluren showing discernable readthrough is 0.01–0.1 $\mu\text{mol/L}$, while the concentration achieving maximal activity is ~ 3 $\mu\text{mol/L}$ [[URL](#)].

Readthrough of premature RNA stop codons by ataluren in cell-based assays was rapidly reversed upon withdrawal of the drug, a fact which demonstrates that only continuous exposure to effective ataluren concentrations can sustain a maximum optimal activity in vitro [[URL](#)].

The [mdx mouse model](#) (a frequently used experimental model for studying DMD, in which a "UAA" PTC replaces a glutamine codon in exon 23 of the dys-gene [[URL](#)]), is used extensively for studying DMD: ataluren treatment of cultured mdx myotubes yielded full-length dys AND ataluren-treated mdx mice had improved muscle function [[URL](#)] [[URL](#)]. A constantly maintained ataluren intracellular concentration in the interval 2–10 $\mu\text{g/mL}$ led to the production of full-length dys, as demonstrated by [western blotting](#), and to partial restoration of membrane-localized dys in cardiac muscle and in all skeletal muscles examined (up to dys levels of ~ 20% that of muscles from wild-type C57BL/6 mice) [[URL1](#), [URL2](#)] [[URL](#)]: this partial restoration was enough for ataluren to improve protection against contraction-induced injury such that the decrement in muscular force/strength was NOT different from that of the wild-type mice [[URL](#)]; consistent with muscle strength/force improvement, the levels of [creatine kinase \(CK\)](#) (an important [rhabdomyolysis](#) serum marker in DMD), were also reduced with ataluren treatment, with all these data suggesting that ataluren significantly decreases muscle fragility.

Important note. PTCs can also be recognized in the nucleus in a reading-frame-dependent manner by a [nuclear PTC recognition system](#): this is a recently discovered layer of proofreading for mRNA and may be vital for ensuring the very high fidelity needed for the [translation](#) of the [spliced](#) mRNA into [proteins](#) [[URL](#)].

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The known pharmacological mechanism of ataluren in detail (2). Ataluren was also shown to be effective in promoting readthrough of a premature RNA UGA codon in synthetic [firefly luciferase \(LUC\) mRNA](#) [URL] in a [reticulocyte cell-free translation](#) system, supporting the initial hypothesis that ataluren actually targets the [translation process](#) and not the [transcription process](#): this ataluren-treated cells (harboring a LUC reporter) DID NOT alter LUC mRNA levels, indicating that neither transcription nor mRNA stability were affected [URL]; ataluren was also demonstrated to NOT HAVE ANY significant effect on the levels of normal mRNAs or mRNAs that are endogenous substrates for the natural substrates of the [nonsense-mediated mRNA decay \(NMD\)](#) [URL]: NMD was demonstrated to be a complex and hugely important process (for all [eukaryotic](#) life forms, which use NMD as a surveillance pathway, to reduce errors in gene expression by eliminating mRNA transcripts that contain PTCs!), a process which also heavily depends on the integrity of the cytoskeleton, as recently demonstrated in a very important study on NMD [URL] (see also this [URL1](#), [URL2](#), [URL3](#)).

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The known pharmacological mechanism of ataluren in detail (3). Ataluren activity did not affect frameshift mutations (FSMs) (meaning that ataluren cannot correct a FSM or a frameshift mRNA-reading error) AND was unable to promote readthrough of multiple sequential PTCs. Ataluren was also demonstrated to be SELECTIVE for PTCs and NOT lead to readthrough of normal TCs: more specifically, ataluren-treated cells (in vitro) DID NOT produce any abnormally elongated proteins; ataluren also DID NOT promote readthrough of normal TCs in vivo (Western blotting could NOT detect any ataluren-induced alterations in readthrough of normal TCs in tissues from rats and dogs, or in [peripheral blood mononuclear cells \[PBMCs\]](#) from human patients) [URL] [URL].

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Important note. Ataluren was demonstrated to NOT produce any clinical improvements in DMD patients who have already lost ambulation (the capacity of walking independently), thus it is not cost effective and NOT INDICATED IMPLICITLY in those DMD patients.

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The main studies of ataluren in patients with DMD caused by ssNSMs (nonsense mutation-caused DMD or “nmDMD”). Ataluren has been evaluated in nmDMD patients in two randomized, double-blind, placebo-controlled trials: one Phase Ib trial [URL] and one Phase III trial (Ataluren Confirmatory Trial in DMD [ACT DMD]) [URL]: both these trials demonstrated the efficiency (over/versus placebo) of ataluren doses of 40 mg/kg/day (with only mild to moderate adverse effects) in a range of functional end points over 48 weeks, with a favorable benefits-to-risks ratio [URL].

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The indications of ataluren. Ataluren is standardly indicated ONLY to DMD patients WITHOUT loss of [ambulation](#) with DMD phenotypes caused by [nonsense mutations](#) (NSMs) in stricto sensu (abbreviated in this paper as ssNSM, with ssNSM being an [in-frame](#) point mutation in a sequence of DNA that results in a PTC at the site of that mutation, or a point-nonsense codon in the transcribed mRNA [at the same corresponding site/point], and in a truncated, incomplete, and usually nonfunctional protein product): ssNSMs occur in ~13% (10-15%) of all known DMD cases [URL] [URL]: for example, employing the frame-shift checker from the Leiden Muscular dystrophy website (www.dmd.nl) revealed FSMs

(out-frame mutations) in ~76% (34 patients) and in-frame mutations in ~24% (11 patients) [URL]. **Important note.** Ataluren is indicated for the treatment of nmDMD ambulatory 5-year old (or older) patients in Brazil, Chile, Israel, the Republic of Korea, Ukraine and Romania: recently the indication has expanded to include 2-year old nmDMD patients (or older) in the European Union (with Romania being an exception however) plus: Iceland, Liechtenstein and Norway; however, it is important to emphasize that the efficacy of ataluren has not yet been demonstrated in those patients who already lost ambulation [URL].

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Other molecules with great perspectives in helping DMD patients with NSMs (and possibly with PTC-associated FSMs) similarly BUT more efficiently than ataluren. There are some new molecules that are currently studied for their property to suppress PTCs (by promoting the PTCs readthrough [PTC-RT]) [URL]: engineered suppressor [transfer RNAs \(tRNA\)](#) [URL, see also this URL] (with still very low efficiency on mouse models but under fervent research), [paromomycin](#) derivatives (of 1st generation: NB30; of 2nd generation: NB54, which both are ~15-fold less toxic than [gentamicin](#); 3rd generation [derived from the [aminoglycoside antibiotic geneticin](#) {G418}]: NB74, NB84 and NB124; 4th generation [derived from [neomycin](#)]: [pyranmycin](#) {TC007}), [clitocine](#) [URL], new PTC-readthrough (RT) compounds (RTCs) (1st generation: RTC13 and RTC14; 2nd generation: GJ071 and GJ072, plus their analogs, namely RTC204 and RTC219); other non-related compounds like: BZs (BZ6 and BZ16), [negamycin](#) (with much lower [cytotoxicity](#) and NO [ototoxicity](#), in contrast with gentamicin), some [macrolides](#) ([tylosin](#), [josamycin](#), [spiramycin](#), [erythromycin](#) and its derivative, [azithromycin](#) which worked at a ~100-fold lower concentration than any other compound tested so far, enabling a significant reduction of the therapeutic dose) and escin (a mixture of [saponins](#) with [anti-inflammatory](#), [vasoconstrictor](#) and [vasoprotective](#) effects found in [Aesculus hippocastanum](#) (the horse chestnut), which escin was proved to induce a significant PTC-RT, both in the in vitro tests and in the cell lines from a [cystic fibrosis](#) patient) [URL].

3. The leading hypothesis of this paper which propose ataluren to be also tested in DMD patients with frameshift mutations associated with isolated premature RNA stop codons

Although ataluren isn't able to correct a frameshift mutation (or a frameshift mRNA-reading error) and cannot promote readthrough of multiple sequential PTCs, it was CLEARLY demonstrated to promote readthrough of single/isolated PTCs NO MATTER their exact position in the exons of the dys-gene: and this is the exactly the case of NOT ONLY NSMs, BUT MAY ALSO be the case of some FSMs associated with possible isolated PTCs at some arbitrary distance from that localized/specific/point-like FSM.

Here are the main arguments in favor of further studies to test ataluren in DMD cases with FSMs with AT LEAST ONE associated PTC (at distance from the site of that FSM), AT LEAST in DMD patients without loss of [ambulation](#) with FSMs located in exons with indexes (i) formally larger than $i=40$ (which $i=40$ is the approximate half of $i_{max}=79$, which i_{max} is the index of the last exon of the dys-gene) (see next)

Argument no. 1 (Arg1). OFTEN, at least in DMD patients with FSMs located in exons with high indexes (formally $i > 40$), the minimal optimal length of dys (for it to reasonably or even optimally couple with both the cytoskeleton [plus cellular membrane] at one end and with the extracellular matrix at its other dys end) IS MORE IMPORTANT than the exact sequence of [amino acids](#) (which are altered by those FSMs starting from that specific i-indexed exon affected/alterd by that FSM). **Example.** This is also the case of one DMD male child-patient (treated by the author of this paper and sequentially published in 2018 and 2019 [1,2,3]) which has a duplication of nucleotide no. 7547 from the 52nd of the dys-gene, which duplication is actually a FSM which wasn't extensively studied by the genetic laboratory in 2016 to check if that FSM is also associated with a PTC at distance from the exact position of that mutation (this is an additional analysis which is NOT routinely done in Romanian genetic labs, although there may be some advantages and important supplementary information offered by this additional analysis/simulation, as argued next). Furthermore, the FSM of this DMD patient occurs in the 52nd exon which assures a length of this abnormal dys significantly larger than the half of the normal dys length (probably measuring $52/79 \sim 2/3$ of the original normal dys): the hypothesis launched by this paper also includes this DMD case, in the sense that, if any PTCs will be discovered in the 52nd exon or 53rd exon (of the dys-gene) for example, a potential ataluren-induced readthrough of such a PTC (in this specific DMD case) will almost surely add some significant supplementary length to his abnormal dys length, increasing the probability for that dys to be more functional than the non-ataluren treated abnormal dys of this DMD patient. **Note.** Of course that there is also the possibility for this DMD patient to have an abnormally large dys, because this FSM may also alter the normal TCs so that the [transcription machinery](#) to transcript the FSM-altered DNA into a much larger mRNA and a much larger dys implicitly (by further [translation](#)).
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Argument no. 2 (Arg2). There is a registry called "STRIDE" (ClinicalTrials.gov identifier: NCT02369731, which includes patients with DMD caused by NSMs: the first drug registry for patients with NSM-caused DMD and the largest real-world registry of its kind to date) which initially included 9 ataluren-treated patients with PTC-associated FSMs: Arg2 is that their treating physicians interrupted ataluren in these 9 patients (which were all excluded from this registry and from the effectiveness analyses) with the rigid (vague and superficial) argument that "these (PTC-associated FSMs) DMD patients are not expected to benefit from ataluren treatment", WITHOUT any separate effectiveness analysis (no rhabdomyolysis status was presented/published) at the end of their ataluren treatment [[URL](#)].
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Important observation. It is NOT excluded that these PTC-associated FSMs to even alter the [\(RNA-\)splicing](#) mechanism (acting on that mutant mRNA transcript), so that the final RNAm product (of that splicing process) to also be altered additionally.
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Proposal of further specific studies to test the main hypothesis launched by this paper (see next).

Study idea no. 1a. This paper proposes a retrospective study to analyze all the known genetically demonstrated DMD cases caused by FSMs and to check which FSMs cases (associated with DMD phenotypes) are ALSO associated with at least one PTC at distance from the site of each DMD-by-FSM case in part. The

results of this genetic study should be also checked in correlation with the demonstrated molecular lengths and [molecular masses](#) of dys in each DMD case in part (by using various techniques applied on dys, like [western blotting/protein immunoblotting](#) or [SDS-PAGE](#) for example, which SDS-PAGE is a variant of [polyacrylamide gel electrophoresis](#), an analytical method in [biochemistry](#) for the separation of charged molecules in mixtures by their molecular masses in a spatially uniform [electric field](#)).
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Study idea no. 1b. Ataluren (or other PTC suppressing molecules, as listed in the previous section) may then be tested in those specific DMD child cases (without loss of ambulation!) with PTC-associated FSMs in exons with indexes $i > 40$ associated with too short dys non-functional or "hypofunctional" dys variants. In other words, Ataluren may be used as a "research tool" to exactly determine if in those DMD cases (caused by PTC-associated FSMs in exons with a sufficiently large positive integer index $i_x > 40$) it is more important or not for that mutant dys to have a larger length (despite the incorrect sequence of amino acids translated from exons with indexes $i > i_x$) THAN to have a correct sequence of amino acids but short dys length.
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Study idea no. 1c. Given the effects of Asea in DMD children (boys) (with preserved ambulation) demonstrated in the first two published case reports [1, 2, 3, 4], Asea and ataluren even deserve double-blind randomized placebo studies in DMD cases caused by PTC-associated FSMs, with the selected cohort of DMD patients organized in 4 groups: (1) one group with placebo, (2) the 2nd group with Asea only, (3) the 3rd group with ataluren only AND (4) the 4th group with Asea plus Ataluren combined to check any possible/potential synergy between Asea and ataluren. **Important note.** Given the high costs of ataluren, the author of this paper will first test the potential synergy between Asea and [escin](#) (an [Aesculus hippocastanum](#) [horse chestnut] extract with similar capacity in promoting PTC-RT: see previous section by using "escin" keyword) in the patient 1st published as a case report [1, 2].

4. The final conclusions of this paper

In a final conclusion (1), ataluren deserves extensive studies on that subgroup of DMD patients with FSMs causing the occurrence of at least one (and preferably just one OR more-but-isolated, NOT in sequential occurrence!) PTC at distance from that FSM (in the same exon or in other subsequent exons)
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In a final conclusion (2), extensive virtual RNA simulations should be done with professional software (like [Gensearch from PhenoSystems](#) for example) in DMD patients with FSMs so that to identify IF and WHERE PTCs may possibly occur (at distance from the site of that FSM) in those selected subgroup of DMD patients with FSMs. It is ideal and even mandatory that these software predictions to be also verified by complementary studies testing the length/molecular weight of dys (usually by [electrophoresis](#)) for each pre-identified case in part.
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In a final conclusion (3), this paper advices geneticians in general to perfect the standard genetic bulletins in these specific PTC-associated FMS DMD cases so that all PTCs associated to each FMS in part to be mentioned on those genetic bulletins (of these DMD patients) together with the predicted lengths and sequences of dys (for each case in part): of course that these

genetic bulletins also have to contain a CD on which the predicted dys sequence (starting from the abnormal amino acid corresponding to the FMS locus until the end of abnormal dys) to be saved/written.

5. References (partially integrated as Wikipedia URLs in the text)

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