Supplementary Online Content

Clemens PR, Rao VK, Connolly AM, et al; CINRG DNHS Investigators. Safety, tolerability, and efficacy of viltolarsen in boys with Duchenne muscular dystrophy amenable to exon 53 skipping: a phase 2 randomized clinical trial. *JAMA Neurol*. Published online May 26, 2020. doi:10.1001/jamaneurol.2020.1264

This supplementary material has been provided by the authors to give readers additional information about their work.

eMethods.

DYSTROPHIN INDUCTION ASSESSMENTS

Dystrophin induction was assessed by Western blot, RT-PCR, MS, and IF staining, as previously described (Uaesoontrachoon et al. 2019). Antibodies used were: anti-dystrophin (ab15277, Abcam, MA, USA), anti-VCL (SPM227; Abcam), anti-ACTN2 (A7811; Sigma, MO, USA), anti-α-SPTA1 (ab139403; Abcam), and anti-LAMA2 (MAB1922; Millipore, MA, USA). For Western blot analysis, all biopsies were run in triplicate gels and normalized to myofiber content in the biopsy using Coomassie blue stain of myosin heavy chains remaining on the gel after membrane transfer and immunodetection of α-actinin on the membrane. Protein levels were assessed using standard curves on each gel (range from 0-25% of normal levels) generated by mixing 5 normal control samples with one DMD sample. For RT-PCR, bands corresponding to specific versions of the spliced dystrophin mRNA were visualized by gel electrophoresis, and the amounts of different mRNA isoforms were compared. Dystrophin peptides were identified and quantified using reversed-phase nanoflow high-performance liquid chromatography with high resolution MS. IF staining for dystrophin was performed on serial muscle biopsy sections in duplicate. The percentage of dystrophin positive fibers and dystrophin intensity were calculated and normalized to laminin α-2 and α-sarcoglycan on serial tissue sections.

SAFETY ASSESSMENTS

Safety assessments included the following:

1. AEs
2. SAEs (defined as ≥1 of the following: death, life-threatening, hospitalization, disability or permanent damage, congenital anomaly/birth defect, or an important medical event that could have jeopardized the patient and could have required medical or surgical intervention to prevent any of the former events)
3. Laboratory test results
    a. hematology
    b. blood chemistry
    c. urinalysis
    d. cytokines
    e. anti-dystrophin antibody
    f. antidrug antibody
4. Vital signs
5. Electrocardiogram
6. Height
7. Weight
8. Physical examination

Uaesoontrachoon K, Srinivassane S, Warford J, et al. Orthogonal analysis of dystrophin protein and mRNA as a surrogate outcome for drug development. *Biomark Med.* 2019;13(14):1209-1225.

eTable 1. DMD variant type

n (%)	Viltolarsen Treatments						CINRG DNHS	
	Study Period 1			Study Period 2				
	Placebo (n=5)	40 mg/kg/wk (n=6)	80 mg/kg/wk (n=5)	40 mg/kg/wk (n=8)	80 mg/kg/wk (n=8)	Total (N=16)	Exon 53 amenable (n=9)	All participants (N=65)
DMD diagnosis amenable to exon 53 skipping								
Yes	5 (100)	6 (100)	5 (100)	8 (100)	8 (100)	16 (100)	9 (100)	9 (13.8)
Single exon deletion	0	0	0	0	0	0	3 (33.3)	3 (4.6)
Multiple exon deletions	5 (100)	6 (100)	5 (100)	8 (100)	8 (100)	16 (100)	6 (66.7)	6 (9.2)
No	0	0	0	0	0	0	0	56 (86.2)
Single exon deletion	0	0	0	0	0	0	0	4 (6.2)
Multiple exon deletions	0	0	0	0	0	0	0	30 (46.2)
Single exon duplication	0	0	0	0	0	0	0	3 (4.6)
Multiple exon duplication	0	0	0	0	0	0	0	3 (4.6)
No large deletion/duplication	0	0	0	0	0	0	0	16 (24.6)

eTable 2. Mean dystrophin induction

Samples		Dystrophin Western blot		Mass Spectrometry	Dystrophin IF Analysis	RNA RT-PCR
Participant Cohort		Normalized to myosin heavy chain	Normalized to alpha-actinin	% Dystrophin	% Dystrophin-positive fibers	% Skipped mRNA (molarity [nmol/L])
		Mean±SD	Mean±SD	Mean	Mean±SD	Mean±SD
40 mg/kg	Pre	0.3±0.1	0.2±0.2	0.5	1.5±1.0	0.0±0.0
	Post	5.7±2.4	5.4±2.8	2.1	14.3±7.8	17.4±7.2
80 mg/kg	Pre	0.6±0.8	0.4±0.7	0.6	1.8±2.4	0.0±0.0
	Post	5.9±4.5	3.7±2.4	4.2	34.8±20.4	43.9±16.7
Overall	Pre	0.4±0.6	0.3±0.5	0.6	1.7±1.8	0.0±0.0
	Post	5.8±3.4	4.5±2.6	3.1	24.5±18.3	30.6±18.5

eFigure. Study design