Table 3 Analysis of time from disease onset to walking with assistance, wheelchair use, and ambulation loss

									Incidence-free proportion	
	Mean	SD	95% CI		Median	Interquartile range	95% CI		10 years	20 years
Aassistive device use	12.4	1.3	10.0	14.9	8.0	5.0-13.0	6.3	9.7	0.38	0.18
Wheelchair use	15.2	0.8	13.6	16.9	14.0	8.0-22.0	11.8	16.2	0.64	0.29
Loss of ambulation	21.1	1.4	18.3	23.8	21.0	11.0-37.0	15.4	26.6	0.78	0.51

non-ambulant participants was higher than that of ambulant participants, although the difference was not significant (non-ambulant 22.0 ± 4.3 vs. ambulant 20.6 ± 4.6, $p = 0.077$). The number of participants who were underweight was greater than that of the normal population. Proportions of men and women who were underweight were 18.2% (n = 11; 16.4 ± 1.9; median, 17.2; range, 12.1-18.5) and 34.8% (n = 23; 16.9 ± 1.3; median, 17.1; range, 13.6-18.4), respectively, and were 4.7% and 9.1% among healthy men and women, respectively. There were fewer obese participants compared to the normal population (Figure 3) [18]. We identified no significant correlations between BMI and other items, with the exception of age ($r = 0.291$, $p = 0.001$).

Cardiopulmonary function

Information on pulmonary and cardiac function was available for 65% (79/121) and 34% (41/121) of participants, respectively. Of those examined, 33% (26/79) had respiratory dysfunction [% forced vital capacity (%FVC < 80)], and two were using nocturnal non-invasive positive pressure ventilation (NPPV).%FVC was significantly correlated with disease duration ($\rho = 0.479$, $p < 0.01$) and serum CK levels ($\rho = 0.573$, $p < 0.01$). None of the participants who underwent ultrasound cardiographic examination had cardiac dysfunction (ejection fraction, 50-82%; fraction shortening (FS), 25-50%). Mean serum CK level was 459.1 ± 355.0 IU/L (median, 202; range, 11–3133).

Bulletin, newsletter, and facilitation of participant recruitment through GNE myopathy registry

We have been publishing bulletins every three months and sending them to participants and doctors who join Remudy. The bulletin includes useful information regarding clinical care, translational medicine, and clinical trials, as well as articles introducing specialists and specialized hospitals for muscle diseases. These contents are also available on the Remudy homepage. Participant recruitment has also started for additional phase I clinical trials via the Remudy GNE myopathy registry homepage [19].

Discussion

To our knowledge, we describe the first patient registry for GNE myopathy in the world. This registry will contribute to the analysis of the natural history of GNE myopathy and aid in the recruitment of participants for clinical trials.

Participants with GNE myopathy were widely distributed throughout Japan, with 1.7 patients per hospital and 1.3 patients per physician in this study. In contrast, there were 5.8 patients with dystrophinopathy (60% of patients with DMD) per hospital and 3.6 per physician in the dystrophinopathy registry. Thus, while patients with GNE myopathy appeared to be dispersed throughout Japan, patients with dystrophinopathy were concentrated in specialized hospitals, given the need for cardiopulmonary care. This indicates that Remudy may serve a very important role in disseminating clinical information to patients with GNE myopathy and their doctors who are dispersed throughout Japan. The patient registry is also useful in that it allows for recruiting patients and resolving data deviation in comparison with analyses by isolated institutions. For example, the age at disease onset in the Remudy-GNE cohort was later than that determined from an analysis of medical records at the NCNP Hospital (26.8 ± 9.0 years). In our previous questionnaire-based study of core muscle disease center patients, we reported a median proportional duration from disease onset to walking with assistance, wheelchair use, and loss of ambulation of 7.0 ± 0.4 years, 11.5 ± 1.2 years, and 17.0 ± 2.1 years, respectively [14], which were all shorter than the durations determined in the present study. We speculate that this discrepancy may reflect the more advanced disease status of patients at neuromuscular disease-specialized center hospitals. Future improvement of Remudy-GNE registry may conclude why these bias were found in this study.

Three (2.5%) of 121 participants had a past history of ITP in our cohort. As the total number of patients with ITP is estimated to be 20,000 in Japan, with an annual occurrence of 3,000 [20], and the Japanese population was 1.27×10^8 in 2013, the prevalence of ITP is expected to be 15.7 per 100,000 (1.57×10^{-2}%). This means that the frequency of ITP among patients with GNE myopathy is 158 times higher than the general population, at least in our cohort.

UDP-GlcNAc 2-epimerase is a major determinant of cell surface sialylation in human hematopoietic cell lines and a critical regulator of the function of specific cell surface adhesion molecules [6]. Thus, alterations in platelets may occur in patients with GNE myopathy. For example, platelets from patients with ITP show increased electrophoretic mobility, reflecting increased sialic acid content [21]. Among the three participants with decreased platelet counts, two had ITP, raising the possibility of a causal

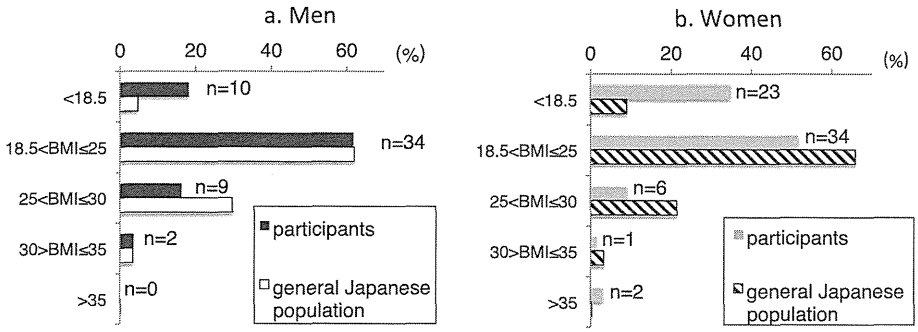

Figure 3 BMI of participants and the general adult Japanese population (aged >20 years). More registry participants were underweight compared to the general population. Proportions of underweight participants were 18.2% and 34.8% for men and women, respectively, and 4.7% and 9.1% for healthy men and women, respectively. There were fewer obese participants relative to the general population. **a.** black column: participants, open column: general Japanese population. **b.** gray column: participants, diagonal column: general Japanese population.

relationship between GNE mutations and ITP, although the underlying mechanisms are unclear and further studies would be necessary to address this issue. Of note, however, this information was obtained based on self-report by patients and/or their families. Thus, the accuracy of the diagnosis is unclear.

It is noteworthy that in some patients, initial symptoms were difficulty lifting heels, but not toes. It is conventionally thought that the initial symptom of GNE myopathy is "foot drop," as tibialis anterior muscles are strikingly affected. Our study suggests that patients whose symptoms start with calf muscle weakness may have GNE myopathy. It is also surprising that some patients had neck and finger weakness from disease onset, despite GNE myopathy being known as "distal myopathy." Thus, GNE myopathy appears to be associated with more phenotypes than expected. However, we are not confident that all patients who chose "difficulty lifting heels" exhibited prominent calf weakness in reality as well, i.e., they experienced greater calf weakness relative to tibialis anterior muscle weakness. This is one limitation of using medical histories and a registration system to collect patient data.

Although we previously reported respiratory dysfunction associated with GNE myopathy [15], 35% of participants in the present study were not examined for respiratory function, indicating that many physicians and neurologists are unaware of the clinical significance of respiratory function in the context of this disease. Although we did not observe any cases of cardiac dysfunction, it may occur in older patients or those with advanced disease. Supporting this is evidence from a study showing that 20% of GNE myopathy mice develop fibrosis in cardiac tissue after 30 weeks of age, with some exhibiting marked endomysial fibrosis, amyloid deposition, and occasionally rimmed vacuoles in cardiomyocytes [8]. This suggests that the risk of cardiopulmonary dysfunction in GNE myopathy should be considered.

In this study, four participants harbored single heterozygous mutations, although they exhibited clinicopathologically definite findings of GNE myopathy. The age at disease onset did not significantly differ between homozygotes and compound heterozygotes. Given that we limited our analysis to all exons and their flanking introns, it is possible that single heterozygotes who exhibited features of GNE myopathy may have mutations in other genomic regions of *GNE*. Yet, in the absence of data using disease-specific biomarkers, it is difficult to distinguish whether these participants had other myopathies and carried a single heterozygous mutation in the *GNE* gene.

Among registry participants, 46% were in the abnormal range of BMI, and the number of underweight participants was markedly higher in both men and women, compared to the normal population. None of the participants or patients of NCNP had dysphagia or other medical problems which might promote weight loss. The BMI of non-ambulant patients tended to be higher than ambulant patients, suggesting that muscle atrophy itself did not cause weight loss. Mechanisms underlying the weight changes may differ from those observed in muscular dystrophy such as DMD [22] or myotonic dystrophy [23], given that obesity was not an issue with most patients with GNE myopathy. It is not clear whether being underweight is beneficial relative to having normal weight in these patients. Prospective analyses will be needed to reveal the relationship between motor function prognosis and body weight.

This study has some limitations worth noting. First, we could not unify the method of grip power assessment. Second, we relied on descriptions of motor function as a crude benchmark for designing clinical trials. Finally, we could not address phenotype-genotype correlations in more depth than was previously reported [9], given the limited number of homozygote patients harboring mutations other than V572L. A larger cohort will be needed to address genotype-phenotype correlations.

Similar to our collaborations involving the dystrophinopathy registry, we are currently in discussions to harmonize the international registry of GNE myopathy of the TREAT-NMD ALLIANCE [ClinicalTrials.gov Identifier NCT01784679, http://www.treat-nmd.eu/gne/patient-registries/international-registry/] (GNE-DMP), in hopes of gaining further insights into the disease. There are two major differences between GNE-DMP. First, as the Remudy aims to establish registration according to genetic diagnosis, inclusion criteria for genetics-based longitudinal natural history studies employing the Remudy-GNE registry require genetic diagnosis (including single heterozygote). Second, we are the only Japanese language registry system. Japan has one of the largest patient groups with GNE myopathy in the world [24]. It is important that patients with this disease receive information in their native language, and that domestic information is supplied for the purpose of Japanese patient accession. Harmonisation would be conducted in order to avoid duplication and double registration of GNE patients while providing the same benefits and opportunities to patients, regardless of where they live. Both registries are similar in their processes utilized for data collection as well as their fundamental ideas regarding the registries, and thus we hope to merge the two registries at some point. According to a tentative agreement, the Remudy-GNE will remain the primary entryway into the international registry as well as serve as the contact site for Japanese patients, and only anonymous data will be stored in the joint data set. Strategies for merging the two registries are currently under consideration.

Our Japanese registry and the TREAT-MND ALLIANCE registry work in close collaboration, and will serve as irreplaceable infrastructures that accelerate research, therapy development, and trial readiness, in addition to increasing opportunities for collaboration and improving global patient care.

Conclusion

The patient registry for GNE myopathy in Japan is useful for gaining a better understanding of the disease, and recruiting patients with genetically-confirmed GNE myopathy for upcoming clinical trials. Further advances and insights can be expected through a soon-to-be-launched international GNE myopathy registry.

Additional files

Additional file 1: Table S1. Genotyping. ED = Glucosamine (UDP-N-acetyl)-2-epimerase domain, KD = N-acetylmannosamine kinase domain. There were more participants who were either homozygous for p.V572L or heterozygous for p.D176V/p.V572L compared to those with other mutations. Although p.D176V was the second most frequent mutation, there was only one participant in the registry who was homozygous for p.D176V.

Additional file 2: Table S2. Allelic frequency. p.V572L was the most frequent mutation.

Additional file 3: Figure S1. Muscle CT of 29 year-old GNE myopathy patient who reported difficulty lifting his heels as one of the first symptoms. Ankle plantar flexion (MMT 2) was prominently impaired (MMT5), and muscle CT revealed that fatty replacement and atrophy were far more prominent in the calf than the anterior part of the lower legs.

Abbreviations

GNE: Glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase; NCNP: National Center of Neurology and Psychiatry; REMUDY: Registry of muscular dystrophies; DMRV: Distal myopathy with rimmed vacuoles; UDP-GlcNAc: Uridine diphosphate-*N*-acetylglucosamine; TREAT-MND ALLIANCE: Translational Research in Europe–Assessment and Treatment of Neuromuscular Diseases; SD: Standard deviation; ITP: Idiopathic thrombocytopenia; FVC: Forced vital capacity; NPPV: Non-invasive positive pressure ventilation; EF: Ejection fraction; FS: Fraction shortening; CK: Creatine kinase.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

MMY drafted/revised the manuscript, conceived of the study, participated in its design, performed data acquisition, data analysis and interpretation, and statistical analysis, and supervised the study. NY conceived of the study, participated in its design, and performed the statistical analysis. YKH drafted/revised the manuscript, performed genetic analysis, and supervised the study. IN conceived of the study, participated in its design, and carried out the genetic analysis. MM drafted /revised the manuscript and supervised the study. EK, HN, and ST conceived of the study and supervised the study. All authors read and approved the final manuscript.

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Long-Term Efficacy of Systemic Multiexon Skipping Targeting *Dystrophin* Exons 45–55 With a Cocktail of Vivo-Morpholinos in *Mdx52* Mice

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Antisense-mediated exon skipping, which can restore the reading frame, is a most promising therapeutic approach for Duchenne muscular dystrophy. Remaining challenges include the limited applicability to patients and unclear function of truncated dystrophin proteins. Multiexon skipping targeting exons 45–55 at the mutation hotspot of the *dystrophin* gene could overcome both of these challenges. Previously, we described the feasibility of exons 45–55 skipping with a cocktail of Vivo-Morpholinos *in vivo*; however, the long-term efficacy and safety of Vivo-Morpholinos remains to be determined. In this study, we examined the efficacy and toxicity of exons 45–55 skipping by intravenous injections of 6 mg/kg 10-Vivo-Morpholino cocktail (0.6 mg/kg each vPMO) every 2 weeks for 18 weeks to dystrophic exon-52 knockout (*mdx52*) mice. Systemic skipping of the entire exons 45–55 region was induced, and the Western blot analysis exhibited the restoration of 5–27% of normal levels of dystrophin protein in skeletal muscles, accompanied by improvements in histopathology and muscle strength. No obvious immune response and renal and hepatic toxicity were detected at the end-point of the treatment. We demonstrate our new regimen with the 10-Vivo-Morpholino cocktail is effective and safe for long-term repeated systemic administration in the dystrophic mouse model.
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Subject Category: Antisense oligonucleotides Therapeutic proof-of-concept

Introduction

Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy, a heterogeneous group of more than 30 genetic disorders characterized by progressive muscle degeneration and weakness.[1] DMD with severe muscle pathology is a fatal X-linked disorder that affects ~1 in every 3,600 live male births.[2,3] Although DMD is caused by various types of mutations in the *dystrophin* (*DMD*) gene, such as deletion, duplication, and nonsense mutations, most patients with DMD have out-of-frame deletion mutations (~65%).[4–6] Becker muscular dystrophy (BMD), a milder form of dystrophin deficiency,[7] mostly results from deletion mutations (~82% of patients) that do not affect the reading frame (in-frame mutations).[4–6] The resulting in-frame transcripts permit the expression of internally shortened but partially functional proteins.[8]

Currently, antisense oligonucleotide (AO)–mediated exon skipping, which can restore the disrupted reading frame by excluding the targeted exon(s), is one of the most promising approaches for the treatment of DMD.[9–13] The aim of exon-skipping therapy for DMD is to slow the disease progression by converting severe DMD symptoms to the milder symptoms seen in patients with BMD. One major challenge to this technique is the fact that individual exons in the *DMD* gene need to be targeted by specifically tailored AOs to cover the majority of patients; thus, the therapy may have limited applicability.[14–16] Another challenge is that the function and stability of each of the resulting short dystrophin proteins are unclear.[17] To overcome these issues, many investigations of multiexon skipping have been conducted in preclinical trials with dystrophic animal models and DMD patient cell lines.[18] In particular, multiexon skipping that targets exons 45–55 at the mutation hotspot of the *DMD* gene has the potential to greatly expand the applicability of exon skipping therapy for DMD patients and produce more stable/functional truncated dystrophin protein. An in-frame mutation that lacks the entire exons 45–55 region has been associated with a milder or almost asymptomatic phenotype in 95% or more of BMD patients with this type of mutation.[19,20] This observation prompted us to investigate multiexon skipping of this entire region. In theory, ~63% of DMD patients with deletion mutations and 45% of all DMD patients could be rescued by skipping the exon 45–55 region according to the Universal Mutation Database (UMD)-DMD database.[19]

Typically, skipping multiple exons is more technically challenging and less efficient than targeting single exons. A new-generation morpholino, the Vivo-Morpholino (vPMO), which possesses a cell-penetrating octaguanidinium dendrimer, has been reported to improve the efficiency of exon skipping.[21–25] Recently, we described a 10-vPMO cocktail (12 mg/kg every 2 weeks) that efficiently induces multiple skipping

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Keywords: antisense therapeutics; Duchenne muscular dystrophy; dystrophin; *mdx52* mouse; multiexon skipping; Vivo-Morpholinos
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of exons 45–55 both *in vitro* and *in vivo* in dystrophic exon-52 knockout (*mdx52*) mice.[26,27] Long-term efficacy and safety of the vPMO cocktail, including recovery of dystrophin-associated proteins (DAPs) and immune response, remain to be determined in the systemic treatment. Patients treated systemically with AO-mediated exon skipping therapy will require repeated administration of AO drugs throughout their lifetime in order to maintain the therapeutic effects. Lower concentrations of AOs that achieve sufficient exon skipping may prevent potential off-target effects associated with long-term systemic treatment.

In this study, we examined the long-term efficacy and safety of multiple exon skipping with a cocktail of vPMOs that target exons 45–51 and 53–55. We demonstrate that nine systemic injections of the cocktail every 2 weeks at a relatively low dose of 6 mg/kg induced bodywide expression of dystrophin and DAPs in the dystrophic skeletal muscles of *mdx52* mice, accompanied by an improvement in pathology, functional recovery, and no detectable immune response and renal and hepatic toxicity by blood tests.

Results

Local injection with the 10-vPMO cocktail at 0.3 μg skips the entire exons 45–55 of dystrophin transcript in the tibialis anterior muscle of *mdx52* mice

To validate the efficacy of exon 45–55 skipping with the vPMO cocktail at a lower dose (a total of 0.3 μg of vPMOs, 0.03 μg of each) than the previous 1.5 μg,[26] we first injected the cocktail into the tibialis anterior (TA) muscles of 8-week-old *mdx52* mice (**Supplementary Figure S1**). AO sequences used are listed in Table 1. An intended skipped mRNA band of 243 bp was detected by reverse transcriptase-PCR (RT-PCR) in the TA muscles 2 weeks after an intramuscular injection with the vPMO cocktail. Sequence analysis showed the boundary of exons 44 and 56, confirming that the entire exons 45–55 region had been removed. In addition to the intended skipped mRNA, intermediate dystrophin mRNAs in which some of the exons were removed were also observed in the nontreated and vPMO-treated samples. These intermediate transcripts were composed of both out-of-frame and in-frame transcripts. In immunohistochemistry with anti-dystrophin DYS1 and P7 antibodies, using a lower dose (0.3 μg), we induced more than 50% of dystrophin positive fibers in treated TA muscle, as well as our previous report with a vPMO cocktail at 1.5 μg.

Table 1 Sequences of antisense oligonucleotides used for a 10-vPMO cocktail to skip exons 45–55

Name	Position	Sequences
45A	+5+29	TGA CGC TGC CCA ATG CCA TCC TGG A
46A	+98+122	CTT TTA GCT GCT GCT CAT CTC CAA G
47A	+22+46	ATT GTT TTA GAA TTC CCT GGC GCA G
48A	-2+23	TTC TCA GGT AAA GCT CTG GAG ACC T
49A	+24+48	AAG CCT TTC CAC ATC CGC TTG TTT A
50A	+48+72	CTG CTT TGT CCT CAG CTC CCG AAG T
51A	+66+90	ACA GCA AAG AAG ATG GCA TTT CTA G
53A	+44+68	ATT CAA CTG TTG TCT CCT GTT CTG C
54A	+23+47	GCC ACG TCT ACA CTT ATC TGC CGT T
55A	+84+108	GCA GTT GTT TCT GCT TCC GTA ATC C

Long-term systemic and repeated injections of the 10-vPMO cocktail induce exon 45–55 skipping and expression of dystrophin and its associated proteins in bodywide skeletal muscles

Next, we examined the exon skipping efficacy of systemic injections with the vPMO cocktail. We performed nine consecutive intravenous injections of the vPMO cocktail, at 6 mg/kg total per injection (0.6 mg/kg for each vPMO) at 2-week intervals, into 8-week-old *mdx52* mice. The experimental term and injection frequency in the systemic treatment were almost twice as much as our preceding study, while the total dosage of 54 mg/kg remained similar to the previously tested dosage.[26] Treated mice did not show any abnormal behavior after the intravenous injections. RT-PCR analysis showed exons 45–55 skipped transcripts averaged 3.5–22.7% in muscles bodywide, including the heart, at 2 weeks after the final injection (Figure 1a). The exon 44 and 56 junction of the intended band was then confirmed by direct sequencing (data not shown). Various intermediate PCR products including out-of-frame and in-frame sequences were observed in different patterns between tissue types and/or individuals after the systemic treatment as well as the intramuscular injection. We also detected dystrophin-positive fibers in all of the skeletal muscles examined by immunohistochemistry with P7 primary antibody (Figure 1b). Western blotting revealed that dystrophin expression levels in all of the tested skeletal muscles averaged 5–27% compared to normal levels in samples from wild-type (WT) mice (Figure 1c). Expression levels of induced dystrophin proteins as well as the expression pattern of the skipped mRNAs varied among different positions within a given muscle sample and among different muscle types. Unlike in the skeletal muscles, immunohistochemistry and Western blotting, respectively, revealed that there were fewer dystrophin-positive fibers and less expression of dystrophin protein (≤2.3% that of WT mice) in the heart muscle after long-term systemic vPMO treatment (Figure 1b,c). In immunohistochemistry with serial sections from biceps femoris (BF) and gastrocnemius (GC) muscles of the treated mice, we observed recovery of DAPs: α1-syntrophin, neuronal nitric oxide synthase (nNOS), α-sarcoglycan, and β-dystroglycan in the dystrophin positive fibers 2 weeks after the last administration with the vPMO cocktail (Figure 2).

Systemic long-term treatment using the vPMO cocktail improves histology and muscle function in *mdx52* mice without inducing detectable immunoreaction

Compared to nontreated muscles, we observed less muscle degeneration and fewer cellular infiltrations in the diaphragm (DIA), BF, quadriceps (QUA), GC, and TA muscles after long-term systemic vPMO cocktail treatment (Figure 3a). We next evaluated detailed histological changes in the muscles of treated mice compared with those in nontreated mice. The percentage of centrally nucleated fibers (CNFs), which is indicative of degeneration/regeneration cycles, was significantly reduced in the DIA, BF, QUA, GC, and TA muscle of the treated *mdx52* mice compared to the nontreated mice, indicating an amelioration of muscle pathology (Figure 3b).

To examine immunoglobulin (Ig) accumulation in dystrophic muscle fibers, which is a hallmark of necrotic fibers due to leaky muscle membranes in DMD patients and *mdx* mice,[28] immunostaining with IgG antibody was performed (**Figure 3c**). Increased IgG signals were observed in endo- and perimysium of DIA, BF, QUA, and HRT of the nontreated and treated mice. The signal intensity and the number of IgG-positive fibers, although not statistically significant ($P = 0.32$ in BF and $P = 0.39$ in QUA, $n = 4$ in NT and $n = 6$ in treated group), were reduced in the treated mice group, indicating improved muscle pathology by the systemic treatment with the vPMO cocktail. Significant improvement in maximum hindlimb grip force was observed in treated *mdx52* mice compared to nontreated *mdx52* mice (**Figure 3d**), although the improvement in the forelimb was not significant (data not shown). To investigate an immune response to long-term administration of the vPMO cocktail, we observed changes in the number of CD3-positive T cells (**Figure 4a**). Although an increase of CD3-positive cells was found in DIA, BF, QUA, and HRT in both nontreated and treated mice, there was no significant difference in the number of CD3-positive cells between the treated and nontreated mice (**Figure 4b**).

Serum biochemical parameters for renal and liver functions are not significantly altered by long-term systemic treatment with the 10-vPMO cocktail

To investigate the potential toxicity of long-term systemic injections of the 10-vPMO cocktail, serum biochemical parameters were tested and statistically analyzed among the groups at the end-point, which was 2 weeks after the final injection (**Figure 5**). A reduction in serum creatine kinase (CK) level, an indicator of muscle damage, was accompanied by histological and

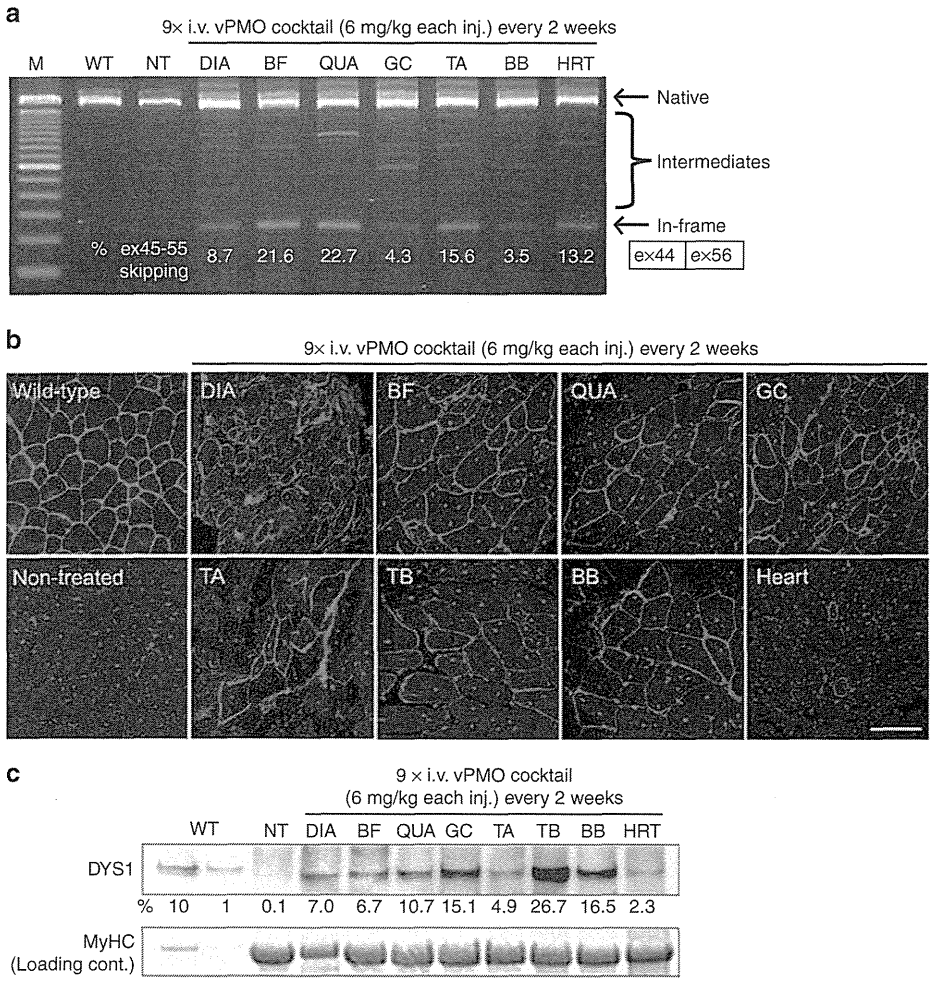

Figure 1 Long-term systemic intravenous injections of the 10-vPMO cocktail. (**a**) Dystrophin mRNAs with exons 45–55 skipped (detected by RT-PCR) in the various muscle types 2 weeks after the last of nine systemic intravenous (i.v.) injections of 6 mg/kg 10-vPMO cocktail (0.6 mg/kg each vPMO) at 2-week intervals. (**b**) Immunohistochemistry with P7 antibody against dystrophin (red) in *mdx52* mice after the treatment. Representative data are shown. Scale bar, 100 μm. (**c**) Western blotting analysis with mouse monoclonal DYS1 antibody after repeated vPMO systemic injections into *mdx52* mice. Truncated dystrophin bands at ~380 kDa (upper panel) are shown in various muscles from the vPMO cocktail–treated *mdx52* mice. Positive controls, 10 and 1% protein (weight/weight percentage) of the TA muscle from WT mice; negative control, TA muscle of NT mice. Myosin heavy chain (MyHC) is shown by Coomassie Brilliant Blue staining as a loading control (lower panel). Representative data from six treated mice are shown. BB, biceps brachii; BF, biceps femoris; DIA, diaphragm; GC, gastrocnemius; HRT, heart; M, 100 bp marker; NT, nontreated *mdx52*-TA muscle; TA, tibialis anterior; TB, triceps brachii; QUA, quadriceps; WT, wild-type C57BL/6J.

functional recovery, but this result did not reach the level of significance. The level of blood urea nitrogen (BUN) in the treated mice was significantly reduced to a level equivalent to that in WT mice. Levels of creatine (Cre), total bilirubin (T-bil), and γ-glutamyl transpeptidase (γ-GTP), a more specific indicator of liver lesions than aspartate aminotransferase (AST) and alanine aminotransferase (ALT) affected by muscle lesions,[26,29,30] were not significantly changed by the long-term treatment. Thus, renal and hepatic toxicity were not detected in the *mdx52* mice after nine consecutive injections of the 10-vPMO cocktail at 2-week intervals.

Discussion

In this study, we demonstrated the feasibility of skipping exons 45–55 in their entirety, which is the mutation hotspot region in the *dystrophin* gene, and rescued expression of dystrophin and DAPs over 18 weeks without any detectable immune response or nephro- and hepatotoxicity in the blood test after nine intravenous injections of the 10-vPMO cocktail at a low concentration of 6 mg/kg. These results indicate that our new regimen for long-term treatment with the vPMO cocktail at 6 mg/kg can safely achieve therapeutic effects similar to our previous study with a dose of 12 mg/kg.[26]

Antisense-mediated exon skipping targeting a single exon is currently close to clinical application for the treatment of DMD, but the multiexon skipping strategy is still far from the clinical trial stage, due to the limited amount of information yielded thus far by preclinical trials.[18] To date, several clinical trials of exon skipping using unmodified morpholinos and 2'O-methylated phosphorothioates (2'OMePSs) are ongoing for DMD. A phase 1/2 clinical trial of exon 44 skipping has been completed and skipping of exons 45 and 53 is being investigated in a phase 1/2a clinical trial conducted by Prosensa (http://www.prosensa.eu/). The exon-skipping phase 3 trial, which targets exon 51 with a 2'OMePS AO, is ongoing.[13] Sarepta Therapeutics is currently conducting a phase 2b clinical trial of exon 51 skipping with a morpholino,[12] while Nippon Shinyaku has started a phase 1 trial of exon 53 skipping (UMIN Clinical Trials Registry number UMIN000010964). Although these targeted exons comprise portions of the hotspot region, skipping of exons 44, 45, 51, and 53 would be applicable to only 6.2% (8.8%), 8.2% (11.8%), 13.0% (19.1%), and 7.7% (11.4%) of all DMD patients (patients with deletion mutations), respectively.[16] In addition to the limitations of therapeutic coverage, another concern is that single exon skipping therapy produces distinct truncated dystrophin proteins depending on patient-specific mutation patterns; this raises a concern that ongoing skipping strategies may not always induce stable/functional dystrophin proteins. By contrast, more than 95% of patients in whom exons 45–55 are deleted in their entirety are reported to have milder BMD symptoms or are asymptomatic.[19,20] BMD is caused by a reduction in the amount functional dystrophin protein.[31] This fact suggests that truncated proteins that lack this specific region are more stable and functionally able to protect muscle tissue from deterioration. It is worth noting that multiexon skipping targeting exons 45–55 would cover ~63% of DMD patients with deletion mutations, according to the UMD-DMD database.[19] Aartsma-Rus's group first tested the concept of skipping of the hotspot region with 2'OMePS cocktails in DMD patient cell lines.[32] Unfortunately, this trial did not achieve sufficient skipping of the intended exons and dystrophin expression. Recently, we demonstrated proof of concept for skipping exons 45–55 with a cocktail of vPMOs; effective expression of dystrophin protein was induced after five consecutive systemic injections at 12 mg/kg every 2 weeks.[26] Positively charged vPMOs have higher cell membrane permeability and improved delivery into muscle fibers. One concern is that vPMOs might have a narrower therapeutic window due to their chemical feature, compared to uncharged morpholinos. Long-term low-dose therapy could be an excellent solution to overcome this

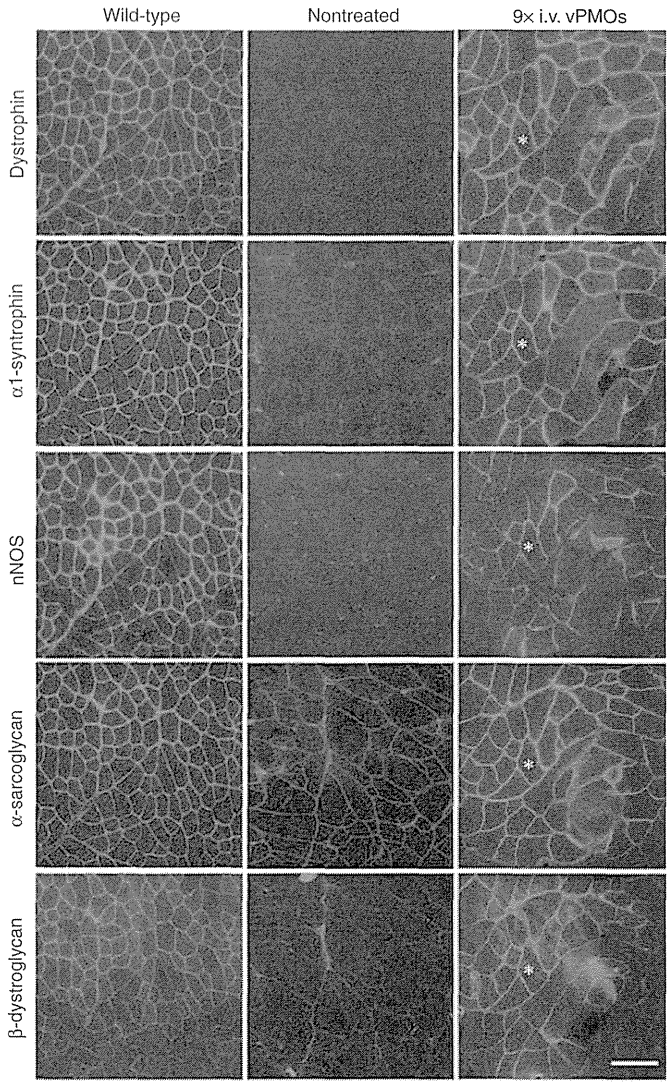

Figure 2 Immunohistochemistry of dystrophin-associated proteins accompanied after 9 times i.v. injections of the 10-vPMO cocktail. Dystrophin, α1-syntrophin, nNOS, α-sarcoglycan, and β-dystroglycan were stained on serial sections of the muscles form nontreated and treated *mdx52* mice. Biceps femoris muscles were shown as representative data in 3 treated mice. Asterisks indicate the same muscle fiber between the images. Scale bar, 100 μm.

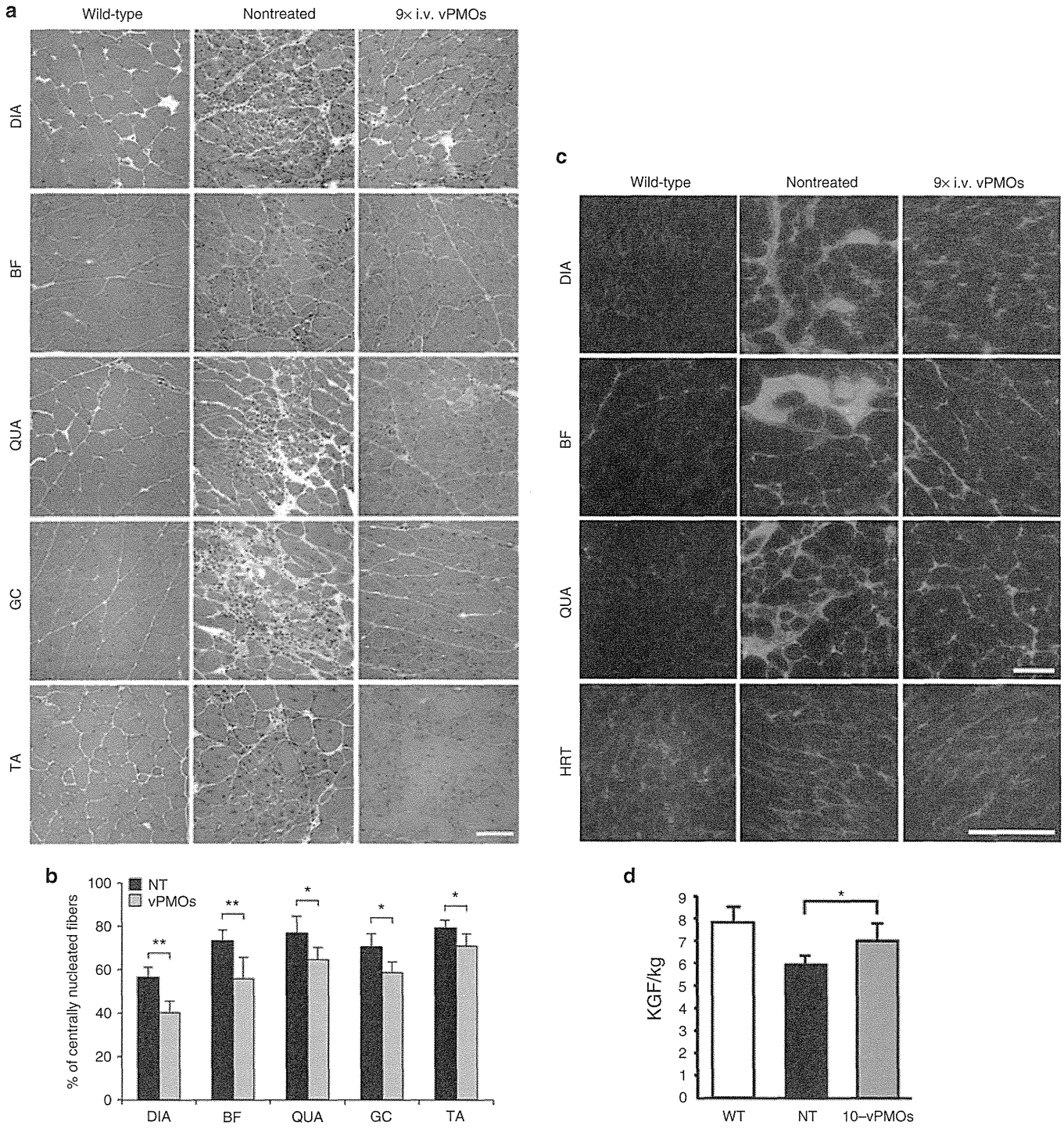

Figure 3 Histopathology and grip strength after systemic injections of the 10-vPMO cocktail. (**a**) Hematoxylin and eosin staining in the diaphragm (DIA), biceps femoris (BF), quadriceps (QUA), gastrocnemius (GC), and tibialis anterior (TA) 2 weeks after the last of nine systemic injections of 6 mg/kg 10-vPMO cocktail at 2-week intervals (9× i.v. vPMOs) compared with wild-type (WT) and nontreated (NT) muscles. Scale bar, 100 μm. (**b**) Significant reduction in percentage of centrally nucleated fibers in skeletal muscles after long-term systemic treatment (n = 4 in nontreated and n = 6–7 in treated group). (**c**) Detection of IgG (green) in DIA, BF, QUA and HRT muscles of WT, nontreated and treated *mdx52* mice 2 weeks after the final injection. (**d**) Increased grip power (kilogram force/kg) of hind limb in *mdx52* mice after eight administrations of the cocktail. Data (n = 5 in nontreated and n = 3 in treated group) are presented as mean ± SD. * $P < 0.05$ and ** $P < 0.01$.

limitation. Thus, this therapeutic strategy has potential for the treatment of ~30 and 45% of all DMD patients based on the LOVD and the UMD-DMD database, respectively.[19,33]

New-generation morpholinos, such as cell-penetrating peptide-conjugated PMOs (PPMOs) and vPMOs, are reported to enable high-efficiency exon skipping at lower doses than

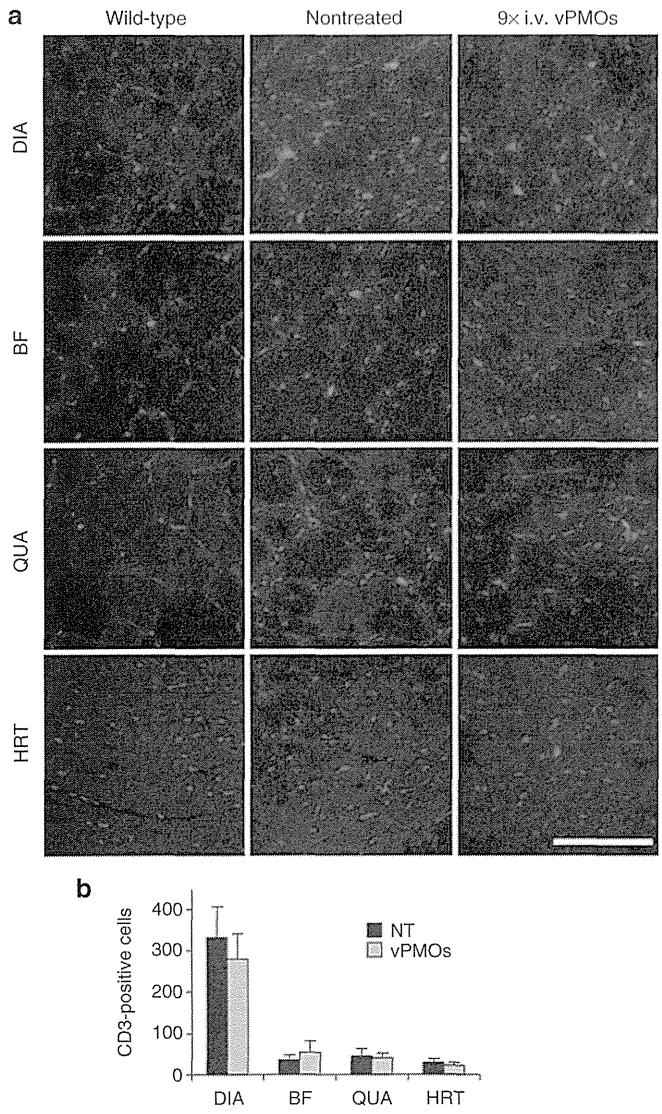

Figure 4 Immune responses against the 10-vPMO cocktail after the long-term systemic treatment. (**a**) Immunostaining against CD3 antigen (red) of a pan T cell marker in diaphragm (DIA), biceps femoris (BF), quadriceps (QUA) and heart (HRT) muscles from wild-type at 6-month-old, nontreated and treated *mdx52* mice 2 weeks after the final i.v. injection of the vPMO cocktail. Nuclei (blue) were counterstained with DAPI. Scale bar, 100 µm. (**b**) Counting CD3-positive cells on the muscle sections. The number of sporadic CD3-positive cells was counted on 10 section areas at random through a 20× objective lens. Data ($n = 4$ in nontreated and $n = 6$ in treated group) are presented as mean ± SD.

unmodified morpholinos and 2'OMePSs.[22,24,27,34] In a previous study, five administrations of a vPMO at a low dose of 6 mg/kg were more effective than a single bolus administration at 30 mg/kg in the *mdx* mouse model.[24] Moreover, intravenous injections every 2 weeks of a PPMO at a dose of 6 mg/kg for 1 year achieved successful dystrophin expression in body-wide skeletal muscles in the *mdx* mouse.[35] In this study, we employed a relatively low dose of 0.6 mg/kg of each vPMO (6 mg/kg in total) and found that long-term treatment with nine injections of the 10-vPMO cocktail at 2-week intervals successfully induced expression of mRNA in which exons 45–55 had been skipped; the result was recovery of dystrophin and DAPs body-wide in *mdx52* mice. Although there were variations in the distribution and expression levels of the skipped dystrophin mRNA/proteins among the different muscle types and/or within positions of a given muscle, and no significant reduction in creatine kinase levels in the treated-mouse group, the amelioration of skeletal muscle pathology and functional recovery that we observed provide evidence of the therapeutic effects of the regimen.

Another concern was that very little dystrophin protein was detected in the heart muscle (≤1% that of WT mice), even though exons 45–55 skipped mRNA was detected by RT-PCR in heart tissue from treated mice. The lower expression of dystrophin protein may be attributed to differences in the posttranscriptional processing for dystrophin generated in skeletal muscle compared to heart muscle. This is supported by a recent study that dystrophin expression level in the heart is higher than in the skeletal muscles and that mutated dystrophin transcripts are less stable than normal transcripts.[36] This could include the rate of decay for the AO-induced dystrophin mRNA and protein, because effective exon skipping in the heart muscle of transgenic mice, which bear target human β-globin intron, has been reported with four intravenous injections of a vPMO at 12.5 mg/kg/day for 4 days as well as in other tissues, including skeletal muscle.[21] Neither bolus nor repeated intravenous/intraperitoneal administration of a vPMO at doses from 15 mg/kg to 30 mg/kg resulted in similar levels of dystrophin mRNA and protein produced in heart muscle compared to skeletal muscles.[24,25] A similar tendency was reported in studies in which 2'OMePSs, morpholinos, and PPMOs were administered to *mdx* mice;[30,35,37–39] moreover, this tendency was also seen in *mdx* mice subjected to adenoassociated virus-mediated exon skipping.[40] In addition, distinct cellular trafficking systems for AOs between skeletal muscle cells and cardiac cells may affect the efficiency of AO-mediated exon skipping in these tissues.[41] Although the processing mechanism of dystrophin transcripts and AO delivery system will need to be more thoroughly characterized in order to increase protein expression, it is encouraging to note that as little as 1–2% dystrophin protein expression in the heart muscle improves cardiac function and pathology in *mdx* mice.[30,42]

A remaining challenge facing the new-generation morpholinos is that there is still insufficient information available about their toxicity. As described above, thus far, frequent systemic intravenous injections at a dose of 6 mg/kg of a single vPMO and PPMO for long-term therapy seem to be an effective regimen with no potential adverse effects in *mdx* mice, which is a rationale for the evaluation of efficacy and safety of the 10-vPMO cocktail at 6 mg/kg in this study.[24,35] Lethal toxicity emerges following single intravenous administration at a dose of 60 mg/kg PPMO in the *mdx* mouse, and 30 mg/kg PPMO systemic injections also cause transient activity reduction in treated mice, indicating potential off-target effects.[35] In nonhuman primates treated by systemic injections with PPMO at 9 mg/kg for 4 weeks, mild tubular degeneration in the kidney has been reported.[34] Although it is speculated that acute lethality/abnormal behavior after an intravenous injection with vPMOs may be caused by multimers clustered by hybridization between their sequences,[43]