

Hereditary inclusion body myopathy: single patient response to *GNE* gene Lipoplex therapy

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Abstract

Background Hereditary inclusion body myopathy (HIBM) is an autosomal recessive adult onset myopathy. It is characterized by mutations of the *GNE* (UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase) gene. Afflicted patients have no therapeutic options. In preclinical testing, we have previously demonstrated the ability to correct *GNE* gene function and the safety of delivery of wild type *GNE* gene using a liposomal delivery vehicle.

Methods A single patient (subject #001) with severe HIBM treated by compassionate investigational new drug received four doses of *GNE* gene Lipoplex via intramuscular injection. *GNE* transgene expression, downstream induction of sialic acid, safety and muscle function were evaluated.

Results Significant durable improvement in locoregional skeletal muscle function was observed in the injected left extensor carpi radialis longus of #001 in correlation with *GNE* transgene upregulation and local induction of sialic acid. Other than transient low grade fever and pain at the injection site, no significant toxicity was observed.

Conclusions Proof of principle for manufacturing of 'clinical grade' *GNE* gene Lipoplex, clinical safety and activity are demonstrated with *GNE* gene Lipoplex. Further assessment will involve intravenous administration and subsequent phase I trial involving additional but less severely afflicted HIBM patients. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords gene; *GNE*; HIBM; Lipoplex; myopathy

Introduction

Hereditary inclusion body myopathy (HIBM) is the term given to a group of adult onset muscle diseases with an autosomal dominant or a recessive pattern of inheritance. These myopathies have variable clinical manifestations but have characteristic muscle pathology similar to sporadic inclusion body myositis, including rimmed vacuoles and tubulofilamentous inclusions but without lymphocytic infiltration [1].

The autosomal recessive form of HIBM is an early adult onset progressive disabling myopathy transmitted through a mutation of the *GNE* gene [2]. *GNE* encodes the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase (*GNE*/MNK), the rate limiting enzyme in the formation of sialic acid [3,4]. Sialic acids are typically found as the terminal sugars on glycoconjugates, where they play pivotal roles in cellular signaling events [5]. HIBM-associated *GNE* mutations (mostly missense mutations) have been shown to reduce sialic acid production [6,7]. Proper folding, stabilization, and function of skeletal

muscle glycoproteins require muscle fiber sialylation [8–11]. *GNE* mutations resulting in hyposialylation of muscle glycoproteins appear to contribute to myofibrillar degeneration and loss of normal muscle function [2].

We constructed a *GNE*-wt-DNA vector using human *GNE* cDNA and the pUMVC3 expression vector and demonstrated transgene expression of *GNE* mRNA and *GNE*/MNK protein in correlation with subsequent increased production of sialic acid *in vitro* [12]. We also complexed the *GNE* expression vector with a cationic liposome, composed of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesterol (*GNE*-Lipoplex) and demonstrated dose related safety in BALB/c mice [13].

We now describe the activity of the *GNE*-Lipoplex in an individual with late stage HIBM in response to multiple intramuscular (i.m.) injections.

Materials and methods

Study design

The subject was assessed for *GNE* gene expression, sialic acid production, and muscle strength prior to treatment with *GNE*-Lipoplex. The extensor carpi radialis longus (ECRL) muscles bilaterally and the left biceps muscle were injected with *GNE*-Lipoplex under ultrasound guidance. The force generation utilizing a break test technique [14–17] of selected muscles including the ECRL and biceps muscle was assessed with a handheld dynamometer (HHD) for muscle strength pre-injection and on days 1, 2 and monthly after each injection. The histopathology and quantitative assessment of *GNE* transgene expression and sialic acid levels of the left biceps muscle was assessed through sequential biopsy performed at baseline prior to injection and on days 3 and 30 after injection.

Prior to injection the ECRL muscle was identified [18,19]: the forearm was fully pronated and a point that was two fingerbreadths distal to the lateral epicondyle was located. Under ultrasound guidance, the ECRL muscle was visualized as the wrist was dorsiflexed with a radial deviation against pressure application to the head of the second metacarpal. The biceps muscle was identified [18,19]: the arm was fully extended with the forearm supinated and a point at the anterior aspect of the mid upper arm was located. Under ultrasound guidance, the biceps muscle was visualized as the elbow was flexed.

The needle was inserted with ultrasound guidance to confirm penetration into the left ECRL and left biceps muscles. An i.m. injection of the *GNE*-Lipoplex was administered into the left ECRL and left biceps muscles and further monitored by ultrasound as solution was visualized spreading proximal and distal to the injection site within the muscle. Four evenly divided injections of *GNE*-Lipoplex (50 µg of DNA suspended in 100 µl of 5% dextrose and water (D5W)/injection for a total of 200 µg per muscle) were administered along the longitudinal axis of the left ECRL and biceps

muscles with a 25-gauge needle. Prior to injection, the subject was premedicated with oral dexamethasone and a combination of intravenous histamine H1 and H2 blockers, oral (p.o.) indocin and p.o. acetaminophen. After the injection of the *GNE*-Lipoplex, the subject was observed for 4 h. During the observation period, vital signs were taken every 30 min for the first hour, then every 1 h until the fourth hour. Vital signs during this period included blood pressure, temperature, pulse, respiration, and oxygen saturation. Toxicities were graded and reported according to the NCI Common Toxicity Criteria for Adverse Events, version 3.0 (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm).

Ninety days after i.m. injection to the left arm, subsequent injections were given to the right ECRL muscle in a similar manner and repeated every 2 months for a total of three injections. This muscle was again assessed with serial strength testing a HHD.

Thirty muscle groups were tested for muscle strength over time, four muscle groups in the neck, two in the shoulders, ten in the legs and 14 in the arms (including the injected muscles) both prior to and after the i.m. injections using a HHD. Muscle groups tested included right lateral neck flexion, left lateral neck flexion, neck flexion, neck extension, bilateral shoulder shrug, elbow flexion, wrist extension, elbow extension, finger flexion, finger extension, finger abduction, thumb extension, hip flexion, knee extension, ankle dorsiflexion, long toe extension and ankle plantar flexion. Isokinetic muscle function testing using a HHD has previously been used to assess quantitative changes in muscle strength [20–25]. Muscle force generation was measured in our study with the Chatillon FCE-200 Digital HHD (Chatillon, Largo, FL, USA). It uses a digital ordinal scale for the measurement of muscle force in pounds, kilograms or newtons. This Dynamometer displays force measurements to the nearest 0.2 pounds to a maximum of 200 pounds for a total of 1000 potential muscle force readings.

Muscle force generation of 30 muscles utilizing the break technique with a HHD was performed pre-injection and on days 1, 2 and monthly after each injection. The break technique requires the examiner to overpower a maximal effort by the subject, thereby producing a measurement of eccentric muscle strength. Ten repeat measurements were taken for each muscle at each time-point. A symptom directed physical examination was performed at baseline and on days 3, 8, 15, 30 of each cycle. Magnetic resonance imaging (MRI) and positron emission tomography (PET) of the injected and contralateral un-injected muscle was performed at baseline and on day 30 (cycle 1 only). Blood studies [complete blood count, chemistry, creatine phosphokinase (CPK), aldolase, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP)] were performed at baseline and on days 3, 8, 15 and 30 of each cycle. Long-term follow-up involved physical exam, blood studies (complete blood count and chemistry) and muscle strength testing at days 60, 90, 120, 150 and 180. Pulmonary function studies and echocardiogram studies

were performed at baseline and at 30 days after the fourth series of injections.

Study population

A single patient with HIBM was enrolled. The inclusion criteria were: (i) the ability to understand and the willingness to sign a written informed consent; (ii) histological and genotypic confirmation of HIBM; (iii) no acceptable effective treatment options; (iv) age >18 years; (v) life expectancy greater than 3 months; and (vi) normal organ and marrow function as defined [leukocytes (>3000/ μ l), Absolute neutrophil count (>1500/ μ l), platelets (>100000/ μ l), total bilirubin (within normal institutional limits), aspartate aminotransferase (AST) (serum glutamic oxaloacetic transaminase)/alanine aminotransferase (ALT) (serum glutamic pyruvic transaminase) (<2.5 \times institutional upper limit of normal), creatinine (within normal institutional limits), creatinine clearance (>60 ml/min/1.73 m² for patients with creatinine levels above institutional normal limits)].

The patient was not receiving any other investigational agents; had no history of allergic reactions attributed to compounds of similar chemical or biologic composition; had no uncontrolled intercurrent illness; was not pregnant or nursing; and was not HIV positive.

GNE-Lipoplex

The expression plasmid design and construction has been described previously [12]. The liposome delivery vehicle is composed of a 1 : 1 molar ratio of two lipids, DOTAP and cholesterol [26]. Liposome size was in the range 350–450 nm and zeta potential was in the range 50–59 mV. The final product was suspended in water plus 5% dextrose (D5W) USP for a total volume of 100 μ l/injection.

The final product consisted of 4 mM DOTAP : cholesterol and 0.5 μ g/ μ l GNE DNA.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) analysis

We utilized a quantitative RNA method of reverse transcription of RNA into cDNA, followed by RT-PCR relative to a known standard to quantify the unknown samples. This method has been previously described [12,13]. Briefly, total RNA was isolated from left biceps muscle biopsy samples by tissue homogenization with the Fast-Prep 24 (MP Biomedical, Solon, OH, USA) and total RNA extracted using the Corbett X-tractor robot (San Francisco, CA, USA) along with the Macherey–Nagel (Bethlehem, PA, USA) NucleoSpin-8 RNA kit. One microgram of total RNA was used as starting material for cDNA synthesis using oligo dT primers and the

Bio-Rad (Hercules, CA, USA) iScript Select kit. GNE-specific primers were designed so that only the plasmid (recombinant GNE transcript, *rGNE*) would be amplified without cross reacting with the endogenous GNE mRNA.

Sialic acid assessment

Individual needle core biopsy of the left biceps muscle and serum samples were used for the quantification of membrane-bound sialic acid by the thiobarbituric acid method [27]. Briefly, samples were resuspended in phosphate-buffered saline and homogenized. The samples were centrifuged and the supernatants were used for Bradford protein estimation and the pellets were used for sialic acid quantification. The amounts of sialic acid were reported as nmol sialic acid/mg of protein.

Sections were deparaffinized and rehydrated. For lectin stains, sections were blocked with 0.1% bovine serum albumin (BSA) and labeled with fluorescein isothiocyanate (FITC)-peanut agglutinin (PNA) or FITC-*Limax flavus* agglutinin (LFA) (EY Labs, San Mateo, CA, USA) for 1 h at room temperature. For poly-sialated neural cell adhesion molecule (PSA-NCAM), antigen retrieval was performed with 0.1 M sodium citrate, and sections were blocked with 1% BSA and labeled with mouse anti-PSA-NCAM (Chemicon International, Temecula, CA, USA) overnight at 4 °C followed by Alexa goat anti-mouse 555 (Molecular Probes, Carlsbad, CA, USA). Imaging was done on an LSM 510 Meta confocal microscope (Carl Zeiss, Oberkochen, Germany).

Histological analysis

Single-needle core biopsies from the injected left biceps muscle at baseline, days 3 and 30 were formalin fixed and paraffin embedded. Slides were prepared, stained with hemotoxylin and eosin, and analysed by a certified pathologist (Propath, Dallas, TX, USA).

Statistical analysis

Statistical analysis was performed on the handheld dynamometer data generated for each muscle group. Separate comparison of the mean muscle strength at each time point was made to the baseline mean for each muscle using Student's *t*-test.

Results

Product manufacturing

GNE-Lipoplexes were manufactured in a Class 10,000 clean room at Gradalis, Inc. (Carrollton, TX, USA) and successfully passed release criteria for sterility, endotoxin, residual manufacturing components, identity,

specificity, and potency (Table 1). Post-production testing was repeated at weeks 1, 2, 4, 8 and 12 to ensure compliance to the same release criteria (Table 1).

The *GNE*-Lipoplexes were stored at 2–8 °C and tested under all release criteria over a period of 3 months (with additional optical testing out to 7 months), demonstrating stability (Table 2). The same batch of *GNE*-Lipoplex was used for all injections. DNA extraction and restriction digest analysis on samples demonstrated that the plasmid DNA was stable and intact within the *GNE*-Lipoplex (Figure 1).

Patient description

The *GNE*-Lipoplex IND was approved by the Food and Drug Administration in July 2008 for a single patient i.m. injection trial. The single patient #001 is a 40-year-old female who developed muscle weakness at age 18 years. She had typical disease progression with loss of muscle mass and function over the subsequent 22 years. Biopsies of the biceps muscle confirmed diagnosis of HIBM by molecular criteria, revealing heterozygosity for *GNE* gene mutations in exon 7 (p.D378Y; c.1183G > T) and in exon 11 (p.A631 V; c.1892C > T). Both mutations have been previously reported in HIBM [28]. The disease specific family history was negative.

Because there was no known effective therapy, the risk of toxic effects from local *GNE* gene expression was felt to be acceptable, in balance with the forearm muscle deterioration expected for an individual with HIBM.

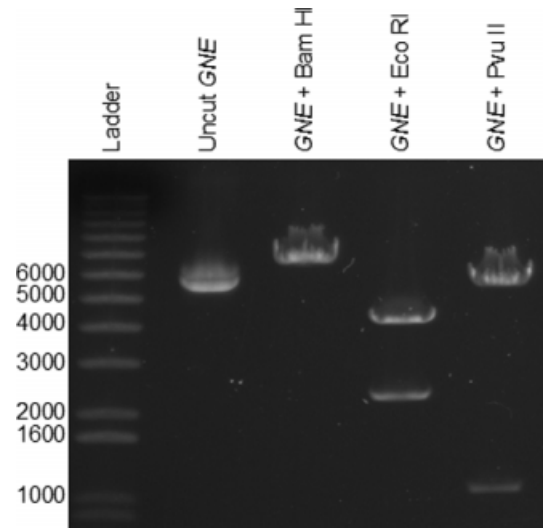


Figure 1. Molecular profiling of *GNE*-Lipoplex. Plasmid DNA extracted from *GNE*-Lipoplex and digested with restriction enzymes. The banding pattern illustrates that the plasmid DNA is intact and stable after 3 months of storage. The uncut plasmid appears to run smaller than 6217 as a result of supercoiled DNA. Enzymes used and expected bands are: *Bam*HI, 6217; *Eco*RI, 2187 and 4030; *Pvu*II, 1028 and 5189

Safety

Treatment was well tolerated. Within 24 h of cycle 1 (left ECRL/biceps injections), minor injection site muscle pain occurred, which responded to analgesic (Tylenol). A low grade fever of 37.7 °C also developed. At 48 h after injection of the left ECRL/biceps, muscle pain was diminished and the fever resolved. Local muscle

Table 1. Final product release specifications and characterization

Release test	Test method	Specification	<i>GNE</i> -Lipoplex values
OD ₄₀₀	Spectrophotometer	0.650–0.950	0.897
Particle size	ZetaSizer Nano	<500 nm	403 nm
Zeta potential	ZetaSizer Nano	> +40 mV	55.1 mV
USP sterility	21 CFR 610.12	No growth	No growth
Bacterial endotoxin	GLP kinetic chromogenic LAL	<5 EU/ml	<0.5 EU/ml
Residual chloroform	Gas chromatography	<75 ppm	0 ppm
DNA banding	Restriction enzyme digest	<i>Bam</i> HI = 6217 bp <i>Eco</i> RI = 2187 and 4030 bp <i>Pvu</i> II = 1028 and 5189 bp	Confirmed
<i>GNE</i> mRNA expression	RT-qPCR	<i>GNE</i> mRNA >1000 fg	62 000 fg

Table 2. Stability test results on *GNE*-Lipoplex

Time point	OD ₄₀₀ (turbidity)	Average size (nm)	Zeta potential (mV)	<i>rGNE</i> ^a (fg)	Sterile	Endotoxin (EU/ml)	Residual chloroform
1 week	0.897	403	55.1	62000	Yes	<0.5	0 p.p.m.
2 weeks	0.887	355	54.7	32500	Yes	<0.5	ND ^b
4 weeks	0.895	387	58.4	30400	Yes	<0.5	ND ^b
8 weeks	0.899	384	55.0	31400	Yes	<0.5	ND ^b
12 weeks	0.893	416	55.2	47000	Yes	<0.5	ND ^b
5 months	0.909	432	50.7	ND ^c	ND ^c	ND ^c	ND ^b
7 months	0.905	438	51.5	ND ^c	ND ^c	ND ^c	ND ^b

^aRecombinant *GNE* expression (transgene). ^bAdditional chloroform testing not done because original sample was within acceptable limits. ^c*rGNE* expression, sterility, and endotoxin testing not performed at 5 and 7 months as a result of a limited amount of product. ND, not done.

ache diminished over the next week. No evidence of recurrent fever or muscle ache was demonstrated after 1 week. Ultrasound of the injection site before and after injection indicated no anatomical alterations. PET and MRI scans before and after injection indicated no change in appearance or fluorodeoxyglucose uptake to the injected muscles. No change in hematologic parameters or electrolytes was noted after injection. CPK, aldolase, ESR and CRP were also normal and not changed from baseline. AST and ALT were slightly elevated (grade 1) at baseline (58 and 70 mg/dl, respectively). There was elevation of ALT to a maximum of 121 mg/dl (grade 2) at baseline of the second right ECRL injection. However, after the second and third injection, ALT lowered to 86 mg/dl (grade 1). AST lowered to 40 mg/dl (grade 0) and bilirubin showed no change from normal. Hepatitis screen was negative. During cycle 2 (right ECRL injections), a maximum temperature of 37.7 °C was observed 4 h after the injection. Transient, Tylenol-responsive, pain at the injection site was again noticed for 7 days; no other adverse events were identified. Pulmonary function and cardiac function was unaltered 30 days after completion of the fourth injection compared to baseline.

Muscle function

HHD muscle strength testing was initiated 448 days before muscle injection to the left ECRL muscle with a drop in force generation from 6.64–3.54 pounds of force (lbf) (preinjection baseline). Since initiation of muscle strength testing, a similar drop in muscle strength was noted in the right ECRL muscle before muscle injection with a drop in force generation from 3.84 (5/29/07)–0.5

lbf (pre-injection baseline). Demonstration of muscle strength over time at preinjection baseline in the left and right ECRL muscles is shown in Figure 2. Results revealed significant increase in left ECRL muscle strength from 3.54 (pre-injection baseline)–4.98 lbf ($p < 0.0001$) within 24 h of injection. Strength declined from 4.98–4.22 lbf ($p = 0.004$) at 90 days post injection, but there was no further significant decline for 150 days, after which values did not differ significantly from baseline. Subsequent injection of the right ECRL also demonstrated a significant increase in muscle strength at 24 h post injection ($p = 0.001$) and 48 h ($p < 0.0001$) from a baseline value of 0.5 lbf. However, muscle strength returned to baseline values at 60 days. The biceps muscle strength (right and left) as tested by the HHD had deteriorated to 0 lbf prior to injection and had no enhancement in muscle strength after injection.

Muscles tested within the left forearm posterior compartment adjacent to the injected left ECRL also demonstrated significant transient increase in muscle strength of short duration (Figure 3).

Furthermore, there was a suggestion that some distal muscles (not adjacent to the left ECRL) with the highest level of baseline residual strength at the time of left ECRL injection (right and left trapezius; right and left quadriceps) also showed transient increase in strength in correlation with *GNE*-Lipoplex injection. The left trapezius and left quadriceps both significantly increased in strength from 16.26–19.26 lbf ($p = 0.00767$) and 15.06–24.36 lbf ($p < 0.00001$), respectively, 72 h after left ECRL injection. The left quadriceps maintained significantly elevated function for 90 days, whereas the left trapezius increase was only observed at 48 and 72 h. The force

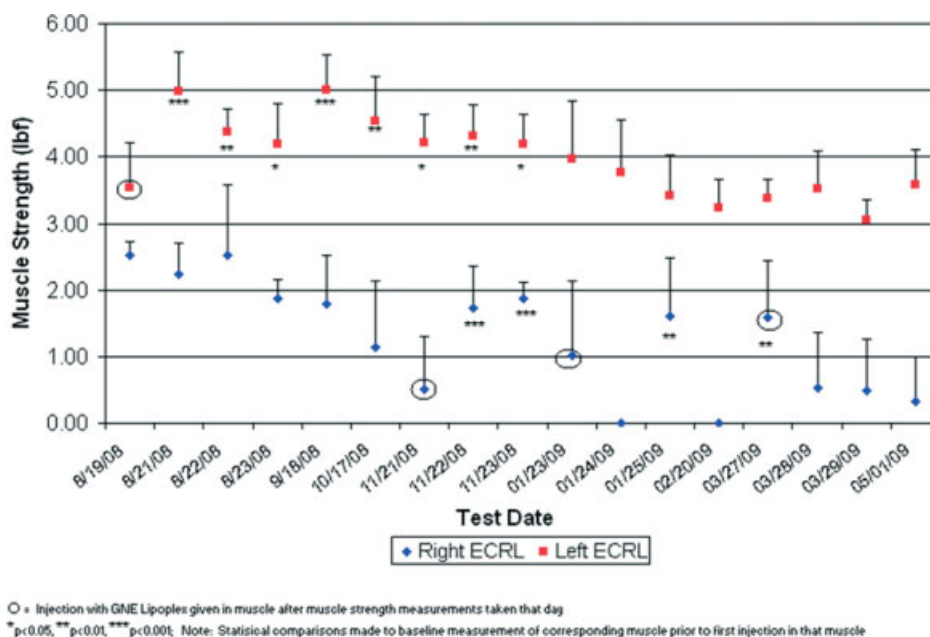


Figure 2. Muscle improvement after i.m. injection in left and right ECRL. The arrow of the left ECRL indicates the time of injection. The red plot illustrates a significant increase in muscle strength 2 days after injection with continued improvement above baseline up to 90 days post injection. The blue plot illustrates the uninjected muscle and its slow decline in strength over time until it was injected (second circle), at which time a significant increase in muscle function is again demonstrated at 24 and 48 h

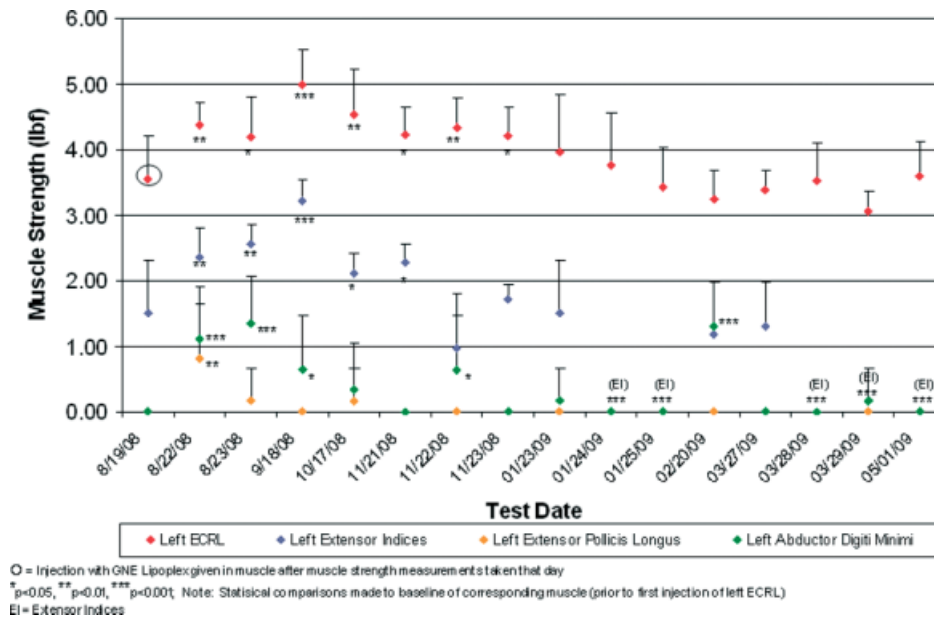


Figure 3. Comparison of left ECRL injected muscle strength to other local forearm muscle strength by dynamometer testing

generation of the right quadriceps and right trapezius did not change in lbf over the same time period, although 24 h after injection of the right ECRL the right trapezius and right quadriceps had a slight increase in strength from 15.66–18.38 lbf ($p = 0.00013$) and 42.01–46.94 lbf ($p = 0.01956$), respectively. This was maintained at significant levels above baseline in the right trapezius for 60 days before returning to baseline. All other distal muscles (not adjacent to the left ECRL) showed a continued loss of muscle force generation throughout the study period consistent with disease progression.

Tissue analysis

Muscle biopsies pre and post i.m. injection in cycle 1 were analysed for GNE mRNA expression, sialic acid concentration, and histopathology. RT-PCR analysis of GNE transgene demonstrated no detectable recombinant GNE (*rGNE*) at day 0. Significant, transient expression of *rGNE* was observed at day 3 but returned to baseline by day 30 (Table 3). Increases in cell surface sialic acid levels were observed at days 3 and 30 compared to day 0 (baseline) (Table 3).

Limits to GNE and sialic acid expression likely resulted from the sample material containing little muscle tissue (5–10%) in the biceps at day 3 and no detectable muscle at day 30 according to pathology reports. This may be attributable to the site of sampling having severe deterioration of the biceps.

We further validated the increase in sialic acid level expression using three antibodies to three different sialic acid dependent proteins (PSA-NCAM, PNA, LFA) on days 0 and 3 from multiple samples of the same muscle biopsy specimens (Figure 4). PSA-NCAM has low expression in the sarcolemma of HIBM2 muscles [10,29]. We observed a low signal at day 0 but, at day 3, the signal was

Table 3. *rGNE* expression and cell surface sialic acid levels in i.m. injected muscle

Time point	<i>rGNE</i> (fg) (n = 4)		Sialic acid (nmol/mg protein) (n = 3)	
	Mean	SD	Mean	SD
Day 0	0	0	22.2	2.1
Day 3	1957	3783 ^a	27.4	0.4
Day 30	0	0	27.6	2.7

^aTwo of the four biopsy samples at day 3 had no low or no detectable GNE mRNA, possibly as a result of the biopsy sites being too far from the original injection site. Hence, the standard deviation is high for the entire day 3 data set.

more intense, as predicted if there was an increase in sialic acid. PNA is a lectin that stains for hyposialylation (especially on O-linked glycans) and will only stain if sialic acid groups are missing and galactose is the terminal sugar on a glycan. It has been shown in the literature that PNA stains intensely on HIBM human muscle tissue [30,31]. In patient #001, there is positive PNA staining at day 0 (indicating hyposialylation), and virtually no staining at day 3, consistent with recovery of sialylation on O-linked glycans. LFA stains for linked/bound sialic acid. Furthermore, there was increased staining at the sarcolemma at day 3 compared to little staining at day 0, indicating more linked sialic acid. The cumulative results from the PSA-NCAM, PNA and LFA staining demonstrate an increased sialylation within the muscle biopsies. These results were reproducible with different sections of muscle biopsy tissue from day 0 and day 3 ($n = 3$). The day 30 sample did not contain muscle tissue and was not analysed. There was no significant difference in sialic acid in the serum, relative to baseline. However, sialic acid levels are low in normal individuals; therefore, detecting a significant change in serum sialic acid is relatively difficult. Histopathology of the biopsied muscle

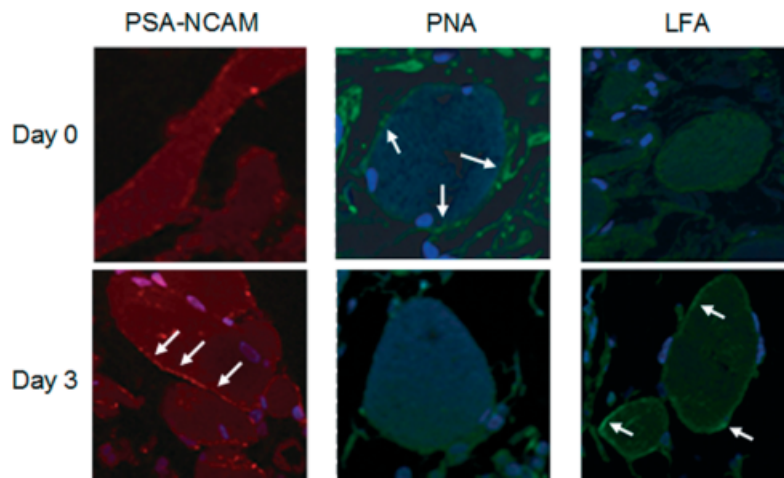


Figure 4. Sialic acid expression in needle core biopsies of patient #001 at days 0 and 3 after *GNE*-Lipoplex intra-muscular injection. PSA is hyposialylated in HIBM muscle. At day 3, there is increased staining at the sarcolemma. PNA stains *O*-linked glycans if terminal sialic acid is missing. Positive staining is shown on day 0 and, at day 3, there is decreased staining overall. LFA stains bound sialic acid and CMP-sialic acid. At day 3, there is increased staining at the sarcolemma. Arrows indicate positive staining for each treatment. The combined staining results indicate an increase in sialic acid production at day 3

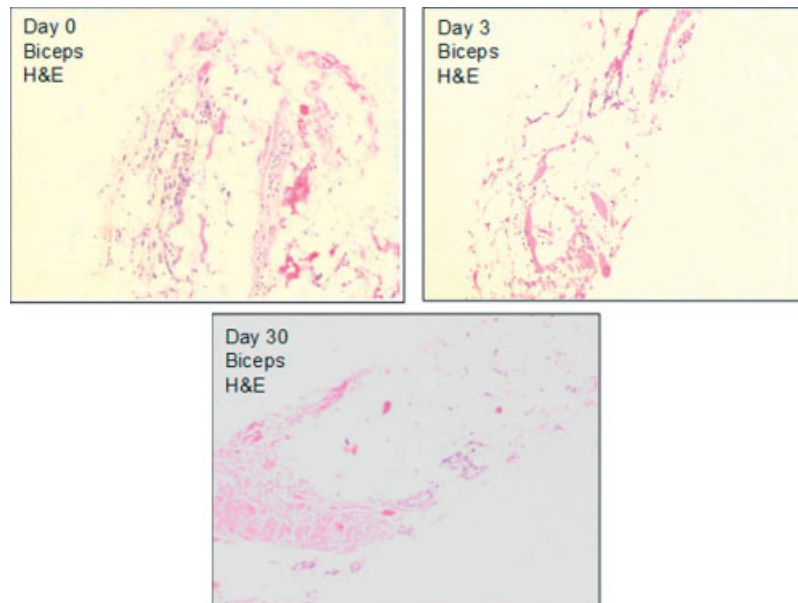


Figure 5. Histology of pre- and post-treatment biceps biopsies. Left biceps muscle biopsies at baseline, days 3 and 30 after injection predominantly composed of fibroadipose tissue, indicative of the severity of stage of disease. Skeletal muscle fibers present appear normal. No inflammatory infiltrate is identifiable at baseline or after injection

revealed no significant inflammatory infiltrate or change in histopathology at days 3 and 30 compared to baseline (Figure 5).

Discussion

We have demonstrated acceptable safety after a series of four sets of i.m. injections of *GNE*-Lipoplex over an 8-month span in a single patient with late stage HIBM. Furthermore, significant transient improvement in muscle strength correlating with transient enhancement of *rGNE* transgene expression and consequent increase of cell surface sialic acid was observed at the muscle injection

site and adjacent compartment muscles. These results are encouraging, particularly given that the dose used for clinical treatment was substantially lower than the maximum tolerated dose involved in animal testing [13], and has already provided evidence to justify and secure orphan drug status. It was surprising, however, that the moderately functional muscle groups including the trapezius and quadriceps muscles improved transiently in correlation with local (left ECRL) *rGNE* transgene expression and increased sialylation, suggesting the possibility of a distant effect. More patients with greater baseline muscle strength and function capacity will need to be treated to fully evaluate the possibility of a distant effect. Our data suggest, however, that prolonged sialic

acid exposure or other unknown GNE induced signaling activity may be a critical issue related to the transient improvement in muscle function. An increase in sialic acid was detected at day 30, although GNE expression was not detectable at the same time frame. The half-life of free and protein bound sialic acid is approximately 3.5 weeks in rat brains [32]. Recycling of sialic acid molecules has been suggested [33] based on the different turnover rates for gangliosides measured after administration of labeled galactose (T 1/2: 10 days) and glucosamine (T 1/2: 24 days), thereby potentially increasing the half life of available sialic acid. It is possible that vector uptake and function in the ERCL where the muscle mass was greater than in the biceps provided a more prolonged expression of the *GNE* transgene. It is not known how long GNE protein, sialic acid protein or other downstream signals are affected. Further assessment of treated patients will be necessary to more accurately describe the molecular dynamics correlating with muscle function improvement.

DNA plasmids can be engineered to provide for long-term gene expression, enhanced gene expression, replication, or integration. Persistence elements, such as the inverted terminal repeats from adenovirus or adeno-associated virus [34,35], have been added to plasmids to prolong gene expression *in vitro* and *in vivo*. These elements appear to bind to the nuclear matrix, thereby retaining the plasmid in cell nuclei. For regulated gene expression, many different inducible promoters are used that promote expression only in the presence of a positive regulator or in the absence of a negative regulator [36]. Tissue-specific promoters have been used for the production of gene expression exclusively in the target cells. Replication competent plasmids or plasmids containing sequences for autonomous replication can be included that provide prolonged gene expression [37]. Other plasmid-based strategies produce site-specific integration or homologous recombination within the host cell genome [38]. Integration of a cDNA into a specific 'silent site' in the genome could provide long-term gene expression without disruption of normal cellular functions. Homologous recombination could correct genetic mutations upon integration of wild-type sequences that replace mutations in the genome. Many improvements can also be made to plasmids aiming to increase their level of gene expression. Viruses enable transgene expression extremely well after entering cells because they take over the transcriptional machinery of the cell to produce their own viral proteins exclusively. However, it may not necessarily be best to take over the entire transcriptional machinery, which would limit homeostatic regulatory control. We have mimicked key elements from 'privileged genes' to enhance gene expression [39,40]. Each of the 0.03% most highly expressed genes produce approximately 2% of the total 300 000–400 000 mRNA transcripts in a human cell [41], and this expression is sustained without shut off. Together, the privileged genes produce approximately 20% of the total mRNA transcripts on average, setting these genes apart as a distinct class of genes. Taking

sequence elements from a privileged gene to include in our expression plasmid, for example, lead to 70-fold increased gene expression *in vivo* [40]. Such an approach may be utilized in a future plasmid design if short-term GNE expression continues to demonstrate muscle function enhancement in further HIBM patients.

GNE/MNK enzymatic activity in HIBM may impair sialic acid production and interfere with proper sialylation of glycoconjugates [2]. Recent data from the Nishino laboratory using the D176V-Tg mouse model demonstrated that muscle atrophy and weakness could be prevented with prophylactic treatment of sialic acid metabolites [42]. These untreated mutant mice show hyposialylation in multiple organs, including skeletal muscle. However, oral treatment with NeuAc or ManNAc improved sialylation and reduced the number of rimmed vacuoles and amyloid deposits. Sialic acid is the only sugar component of glycoproteins that bears a net negative charge. This charged sugar provides the terminal carbohydrate on a variety of *N*-linked and *O*-linked glycans of proteins, as well as glycolipids that mediate cell-cell and protein–protein interactions [5,43].

Although *GNE* mutations are widely accepted as the root cause of HIBM, there is some suggestion that GNE plays a role in functions beyond sialic acid synthesis. Upon nocodazole treatment to inhibit intracellular trafficking, GNE was redistributed from the Golgi to the cytoplasm. This suggests that GNE may play a role as a nucleocytoplasmic shuttling protein [44]. GNE has been associated with α -actin [45] and GNE splice variants have been identified, but their functionalities are yet to be determined [46]. Myoblasts and lymphoblastoid cell lines derived from HIBM patients containing the M712T mutation had reduced epimerase activities, but did not display reduced membrane bound sialic acid *in vitro* [47,48]. However, clinical samples from HIBM patients demonstrated clear reduction in sialic acid levels from muscle tissues [8,10]. GNE is ubiquitously expressed in all tissues, although at relatively different levels in each specific tissue. Krause *et al.* [49] found that GNE protein is expressed in skeletal muscle at equal levels in HIBM patients and normal control subjects. Furthermore, immunofluorescence detection of GNE did not reveal any mislocalization of GNE in skeletal muscle of HIBM patients. Thus, most in the field conclude that impaired GNE function, not lack of expression, is the key pathogenic factor in HIBM [49]. Indeed, Penner *et al.* [7] characterized several different *GNE* mutations and demonstrated that unique mutations altered activity of GNE enzyme to varying degrees of severity, as assessed by downstream enzyme kinetics of ManNAc phosphorylation using a radiolabeled phosphate assay. Interestingly, all mutations did retain a minimal amount of activity relative to the wild-type GNE enzyme. Savelkoul *et al.* [50] suggested that HIBM defects in sialylation may appear gradually and tissues such as muscle, which normally express low levels of GNE protein, are more sensitive to disruptions in the GNE enzyme and hence reduced sialic acid expression.

Hypoglycosylation of α -dystroglycan, a central protein of the skeletal muscle dystrophin–glycoprotein complex, could comprise one mechanism that may provide an explanation for the muscle weakness of patients with GNE mutations. α -Dystroglycan helps to anchor the extracellular matrix to the cytoskeleton of the sarcolemma. If hypoglycosylated α -dystroglycan is unable to properly bind to the extracellular matrix, the connection between the extracellular matrix and the cytoskeleton provided by dystroglycan is lost and unable to stabilize the sarcolemma during contraction-induced stress. Huizing *et al.* [8] examined the glycosylation status of α -dystroglycan in muscle biopsies of four individuals with hereditary inclusion body myopathy. All four muscle biopsies showed absent or markedly reduced immunolabelling with two different antibodies (VIA4 and I1H6) to glycosylated epitopes of α -dystroglycan. These findings resemble those found for other congenital muscular dystrophies, suggesting that HIBM may be a ‘dystroglycanopathy’, and providing an explanation for the muscle weakness of patients with HIBM.

The above findings emphasize that there is more to be learned about the cellular site of pathology and the mechanism of muscle cell degeneration in individuals with HIBM. Many factors are not explained by our work and that of others, particularly the late onset of disease. To further analyse these pathways, efforts to study HIBM mouse models are ongoing [2], as well as a further exploration of the effects of GNE-Lipoplex administration to a HIBM mouse model.

In summary, we have demonstrated proof of principle that local replacement of normal GNE gene and consequent local sialylated glycoconjugate expression can safely and positively affect muscle function in a single individual with HIBM. Further studies will be necessary to fully characterize GNE protein expression and enzymatic activity. Development of therapeutic GNE-Lipoplex is underway and will incorporate an evaluation of the safety and effectiveness of intravenous delivery in additional animal models and in less severely affected individuals with HIBM.

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