B-Cell Depletion is Protective Against Anti-AAV Capsid Immune Response: A Human Subject Case Study

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Abstract

Gene therapy strategies for congenital myopathies may require repeat administration of adeno-associated viral (AAV) vectors in response to several limitations inherent to the clinical design: 1) administration of doses below therapeutic efficacy in patients enrolled in early phase clinical trials; 2) progressive reduction of the therapeutic gene expression over time as a result of increasing muscle mass in patients treated at a young age; and 3) a possibly faster depletion of pathogenic myofibers in this patient population. Immune responses triggered by the first vector administration, and to subsequent ones, represent a major obstacle for successful gene transfer in young patient population. Anti-capsid and anti-transgene product related humoral and cell-mediated responses have been previously observed in all preclinical models and human subjects who received gene therapy or ERT treatment for congenital myopathies. Immune responses may result in reduced efficacy of the gene transfer over time and/or may preclude for the possibility of re-administration of the same vector. This study presents a case of Pompe patient dosed with an AAV1-GAA vector after receiving Rituximab and Sirolimus to modulate the immune responses. A key finding of this single subject case report is the observation that B-cell ablation with rituximab prior to AAV vector exposure results in non-responsiveness to both capsid and transgene, therefore allowing the possibility of repeat administration in the future.

This observation is significant for future gene therapy studies and establishes a clinically relevant approach to blocking immune responses to AAV vectors.

Keywords

AAV; Immunology; Capsid antibody; Pompe disease

Introduction

Pompe disease is a progressive and often fatal neuromuscular disorder resulting from mutation in the gene for acid alpha-glucosidase (GAA), an enzyme necessary for the degradation of lysosomal glycogen within the lysosome. The condition is characterized by a spectrum of disease severity resulting from variable levels of GAA and possibly differential cellular rates of glycogen synthesis. The result of GAA deficiency is an extensive glycogen accumulation in all tissues, especially striated muscle, smooth muscle and the central nervous system (CNS) [1-3]. The range of disease severity encompasses the fatal early-onset form presenting in infancy to a milder adult-onset of disease symptoms. The disease prevalence has been estimated to be 2-4,000 patients in the developed world and incidence is approximately 1 per 40,000 births [4,5]. The phenotypic continuum is directly related to the extent of residual enzyme deficiency [2,6] with complete or near complete deficiency of functional GAA protein in early-onset disease and up to 20% of wild-type activity in late-onset patients [7,8].

Respiratory and skeletal muscle weakness is a key progressive feature of Pompe disease. Respiratory muscle weakness often leads to the need for assisted ventilation and is the principal cause of mortality in early and late onset Pompe patients [9,10]. Skeletal muscle weakness primarily affects the lower limbs and results in loss of ambulation and wheelchair dependency [11]. Generally, GAA activity <1% of wild-type level correlates with presentation in infancy, and 2%-20% GAA activity is seen in later-onset disease [4,5]. Approximately 25% of infants with <1% GAA activity have no detectable GAA protein by Western blot analysis and are considered cross-reactive immunologic material (CRIM)-negative [12]. In CRIM-positive patients, the presence of residual GAA protein usually correlates with a lack of neutralizing antibodies (Nabs) against GAA after initiation of enzyme replacement therapy (ERT). In contrast, CRIM-negative patients lack tolerance to GAA protein and produce a robust humoral immune response to ERT, reducing the efficacy of treatment. CRIM-negative patients receiving ERT have a poor prognosis and diminished survival if not managed with immunosuppression [13-16].

ERT has extended the survival for early onset patients; however, a successful gene therapy strategy may provide additional long-term benefits and improvement in quality of life. Recombinant adeno-associated viral vectors (rAAV) are widely used gene therapy agents for the treatment of genetic diseases. AAV has been used in several clinical trials for the treatment of different conditions including Leber's congenital amaurosis [17,18], hemophilia B [19,20], Pompe disease [21], Sanfilippo syndrome [22], lipoprotein lipase deficiency [23,24], Alpha-1 antitrypsin deficiency, [25], and Limb-girdle muscular dystrophy [26,27]. A critical challenge for the success of gene therapy is the host immune responses to both the vector capsid and transgene product. These immune responses raise concerns regarding the safety and longevity of gene expression. In addition, induction of antibodies by natural exposure to AAV is frequent early in life and may influence the use of AAV as a gene therapy vector [28,29]. This may be critical in developing effective therapeutic strategies for congenital myopathies that may require repeat administration of AAV vectors. Repeat AAV administration may be necessary because low doses, or doses below optimal therapeutic threshold were provided in early phase studies. Further, many subjects may require re-

dosing later in life, as increasing muscle mas or loss of copy number with age may reduce transgene expression. Therefore, potent humoral and cellular memory responses to AAV may compromise the subsequent use of the same vector [28,29]. For these reasons, we are developing strategies to manage these immune responses as a sustainable approach to deliver a safe and long-term expression of a therapeutic gene by AAV-mediated gene therapy.

Humoral responses to ERT observed in Pompe disease include activation of antigen-specific CD4+ T helper cells and production of neutralizing (NAbs) and non-neutralizing (non-NAbs) antibodies. NAbs have been the focus of gene therapy immunology studies because of their effect on diminishing the efficacy of AAV-mediated gene therapy. NAbs bind to the AAV capsid and may block or reduce the transduction of target cells. Additionally, anti-transgene antibodies may develop against the therapeutic protein or may serve as co-activating factors for cell-mediated immunity, possibly leading to elimination of transduced cells [28,29].

One of the strategies to overcome the presence of Nabs in rAAV-mediated gene therapy is pharmacological clin modulation of the humoral immune response. Rituximab is a monoclonal antibody that induces B-cell depletion by binding the CD20 protein found on the surface of B-cells. Rituximab is FDA approved in the treatment of chronic lymphocytic leukemia, non-Hodgkin's lymphoma, and rheumatoid arthritis. Recent studies also showed that Rituximab is able to improve immune tolerance induction in patients with hemophilia [30-32]. In addition, Rituximab was successful in reduction of existing anti-rAAV NAbs in humans receiving rituximab for rheumatoid arthritis [33] and in non-human primates [19,34]. Rituximab's mechanism of action includes complement-mediated cell lysis, antibody-dependent cell-mediated cytotoxicity and direct induction of apoptosis by binding to CD20 [35].

Rituximab may be used in combination with a B-cell and T-cell agent, such as Sirolimus [36]. Sirolimus inhibits the response to interleukin-2 by binding the FK-binding protein 12 and the Sirolimus-FKBP12 complex acts as an inhibitor of the mammalian target of rapamycin (mTOR) pathway and has been used in preclinical studies for gene therapy [37-39]. We hypothesize that immunomodulatory regimen with Rituximab and Sirolimus may be beneficial to control expression of immune responses following gene therapy approaches in humans.

Elder *et al.* [13,15] showed that an immune suppression protocol based on B-cell depletion with Rituximab and T-cell immune modulation with Sirolimus before initiation of ERT in CRIM-negative infants with early-onset Pompe disease was able to block GAA antibody responses and improve clinical outcomes. In this case report, we describe the immunological and clinical consequences of B-cell depletion and T-cell immune modulation in a cross-reacting immunologic material-negative (CRIM-) child with Pompe disease, who was receiving Rituximab and Sirolimus prior to and during enrollment of a phase I-II clinical trial of intramuscular administration of an rAAV1-CMV-hGAA vector protocol [21].

Results

Subject characteristics

The subject of this case study is a CRIM- female child who was diagnosed with Pompe disease (nonsense mutation exon 10, p.W516X; exon 18, p.G828_N882del) at 5.5 months of age. CRIM status was evaluated at the Powell Gene Therapy Center (Gainesville, Florida) by Western blot analysis of skin fibroblasts. The patient presented a severe phenotype with GAA activity <1%, cardiac hypertrophy and ultimately required assisted ventilation. Immediately after diagnosis she was enrolled in a study for which she received ERT and immune modulation for management of antibody response as part of the prescribed treatment regimen. Details of this study are presented in Elder *et al.* [13]. At 45 months of age, the patient was enrolled in another study evaluating the effect of rAAV1-CMV-hGAA intramuscular gene transfer to the diaphragm. Details of this study are presented in Smith *et al.* [21]. Figure 1 summarizes the events of this case study.

Immunosuppression protocol and enzyme replacement therapy (ERT)

At 5.5 months of age, about 20 days before starting ERT, the patient received 375 mg/m^2 of rituximab and 10mg/kg of methylprednisolone intravenously (premedication) weekly for 3 weeks. After the Rituximab induction doses, the patient received daily oral Sirolimus (0.06-1 mg/m^2/day). The dosage was adjusted in order to maintain a Sirolimus serum trough level of 3-7 ng/ml. Once the induction dosage of Rituximab was completed, the patient started receiving a monthly dose of 500-1000 mg/kg of IV immunoglobulin (IVIG) (Gamunex; Talecris Biotherapeutics, Research Triangle Park, North Carolina or Privigen; CSL Behring, King of Prussia, Pennsylvania). The amount was adjusted in order to maintain a trough serum IgG level of 700-1000 mg/dL. The patient continued B-cell depletion regimen with Rituximab at 12 weeks intervals while receiving Sirolimus daily. At 6 months of age, after initiation of B cell depletion, the patient started on ERT infusion at a dose of 20 mg/kg every 7-10 days. The ERT infusion interval was subsequently increased to every 10 – 14 days once clinical improvement was demonstrated.

rAAV-mediated gene therapy

The patient was enrolled in a Phase I/II study to determine the safety and ventilatory outcomes following intramuscular administration of rAAV1-CMV-hGAA (NCT00976352; ClincalTrials.gov). AAV dosing was accomplished at 45 months of age via bilateral diaphragmatic delivery using video-assisted thoracoscopy. Methylprednisolone (10mg/Kg) was given at the time of the procedure up to post-operative day 3. The vector was injected in each hemidiaphragm in three sites, corresponding to the anterior, lateral and posterior costal regions. Each site received 8.33 x 10^{11} vg in 0.8 ml. The total dose was 5×10^{12} vg of rAAV1-CMV-hGAA [21].

Anti-rAAV1 and anti-hGAA circulating antibody and T-cell-mediated responses—Serum samples were assayed by ELISA for circulating antibodies to the AAV1 capsid proteins. The case patient was compared to an immune-modulation control group, which consisted of five children with Pompe disease that received 1-5 $\times$ 10^{12} vg/kg of rAAV1-CMV-hGAA but did not receive immunosuppression regimen. The control group

developed at least a 155-fold increase in anti-AAV1 Ab titer post exposure to AAV1. The patient of this report had no response to AAV capsids through day 180 (Figure 2). The baseline and subsequent titers were at least 2 logs above the limit of detection for the antibody assay.

Enzyme linked immunospot (ELISpot) assay and antigen specific response assay (ASR) were used to test CD4+ and CD8+ T-cells for reactivity against both the AAV capsid and the transgene products. ELISPOT assays to AAV1 capsid protein peptide pools and ASR (Figure 2) to intact AAV1 capsids were unchanged from baseline to day 180. Together, these findings demonstrate the potential to block humoral and cellular immune response following exposure to AAV1. The described regimen facilitated successful regional gene transfer with no adverse events and resulted in a clinical benefit.

Clinical pathology laboratory evaluation.—Safety laboratory data is shown in Table I. Serum IgG was maintained above 700-1000 mg/dL by regular monthly infusions of IVIG. The approach had no impact on WBC, nor was there any evidence of thrombocytopenia or anemia. Serum transaminase levels are commonly elevated in Pompe disease [40] and were unchanged in the patient, from baseline through the 90 days following rAAV1-CMV-hGAA dosing. NT-pro BNP, CPK and CPK-MB were responsive to initiation of ERT and were not influenced by the immune management. There was no impact on CD3 positive lymphocytes. Evaluation of CD20 counts confirmed complete depletion of B-cells by Rituximab.

There were no adverse events identified during the follow up period to date related to the study procedure or immune modulation regimen.

Functional measures—Maximal Inspiratory Pressure (MIP) remained stable at day 90 and increased 6% of baseline value at day 180. Motor Function Measure (GMFM-88) increased 2.95% at day 90 and decreased 1.33% at day 180 (Table 2), which are not clinically significant improvements.

Discussion

The aim of this single subject case report is to describe the observation that B-cell depletion with rituximab prior to AAV vector exposure resulted in immune non-responsiveness to rAAV-CMV-hGAA vector administration, therefore allowing for the possibility of repeat administration of the a vector of the same AAV serotype. This observation is significant for future gene therapy studies and establishes a clinically relevant approach to blocking immune response to AAV vectors. The eye and the central nervous system are considered immune-privileged compartments because of the limited immune responses [29]. However, this approach can be applied to prevent immune response to AAV vectors administered systemically or targeted to cardiac and skeletal muscles or liver.

In a recent review, Mingozzi and High suggested that in muscle and liver gene transfer, the magnitude of T-cell responses against the AAV capsid appear to be correlated to the dose of vector administered [29]. While it is difficult to compare vector dose and capsid load across studies and vector preparations, we can compare the immune response of the subject who

received immune modulation to the control group in this study. In our study, the vector dose for this case subject was 5-fold higher than the control cohort, yet no capsid-specific T cell responses were observed. Since the vector dose in this study is based not based on body weight it is difficult to compare the impact of the immune modulation regimen on dose to T-cell response. The current immune modulatory regimen, which targets B and T cells, may have blocked an anti-capsid response. It is also possible that the young age of this patient contributed to a lack of memory CD8^{+} T cells to capsid, which are believed to be the main cause of the previously observed responses [41,42]. All subjects were negative for capsid antigen-specific response; therefore we cannot conclude that T-cell non-response is due solely to the immune modulation regimen or the low dose of vector. Nonetheless, the impact of B cells, which function both in antibody production and antigen presentation to T cells, on T-cell response is an aspect of the current observation that warrants further study. In this subject, the ELISPOT assay for both capsid peptides as well as for the transgene were negative at all time points. One consideration is that this subject continues to receive the recombinant ERT that originally required for clinical management of Pompe disease. Therefore, ongoing rituximab infusions are administered every 4-6 months to maintain the B-cell depletion. If capsid proteins were persistent past the window of B-cell depletion for a single dose of rituximab, then the anti-capsid response would potentially be observed once the CD20+ B-cell counts were reestablished. While we cannot evaluate that possibility in this patient, a related study was done with AAV9 dosing in non-human primates and capsid antigens did not persist past the period of B-cell depletion [43].

In addition, the subject received supplemental IVIG to maintain a trough serum IgG level of 700-1000 mg/dL. We considered the possibility that pooled sera for production of IVIG might have anti-AAV antibodies represented in the population. Interestingly, the product used in this subject was selected because of the young age of the subject. We tested an aliquot of the IVIG used in this case and there was no detectable anti-AAV1 antibody. Since IVIG that is preferred for pediatric patients is usually obtained from CMV negative donors, we speculate that CMV status may be related to the magnitude of anti-AAV titer.

A similar study with non-human primates was done by administering rituximab and cyclosporine A prior to AAV8 factor IX vector administration [34]. They analyzed the impact of this drug combination on anti-factor IX antibody titer and also observed a decrease in anti-capsid antibody titer. A second dose of vector was given under these circumstances, however the capsid used was AAV6 [34]. Changes in anti-AAV titers have also been studied in the use of rituximab for treatment of rheumatoid arthritis [34]. Patients with low starting titers had a fall in anti-AAV titers to less than 1:400, however, those with high titer at the start of rituximab therapy had no change. This finding supports the use of preventative therapy as described in this report, since plasma cells can be long-lived. In the situation of pre-existing immunity, it may be necessary to consider plasmaphoresis [44] or use of proteasome inhibitors, which would reduce antibody production in plasma cells [45]. The clinical vector used in this study was not further fractionated by density gradient to reduce empty capsids , therefore, a greater proportion of the total capsids are empty capsids. Even with high capsid dose, the antibody response was prevented using the current regimen.

Sirolimus was also used to promote the survival and expansion of regulatory T cells while allowing for programmed cell death of activated effector T cells. Our preclinical [46] and clinical experience [13] supports the combination of Rituximab and Sirolimus as successful immune modulation approach for this patient population.

While this report is a preliminary finding related to a concomitant medication use for ERT, we believe these findings emphasize the need for additional comprehensive prospective studies. To that end, we have begun an IND-enabling, well-controlled non-human primate (NHP) study, which will evaluate the requirements to establish both primary and subsequent dosing with AAV9 vectors, including dose, route of administration and timing of the immune-modulation. The NHP study is an IND-enabling toxicology study that has been proposed to the FDA in support of a human study to directly test the safety and utility of this approach by intramuscular or systemic administration routes.

The clinical application of this approach will ultimately be most important in pediatric patients who will be the most likely candidates for multiple dosing events over the course of their lifetime. Three basic principles support this conclusion. First, the body mass will increase up to 20-fold from infancy to adulthood.. Second, many of the conditions for which early rAAV-mediated gene therapy is being considered have increased rates of cell division or cell death due to the underlying disease. We have observed loss of vector genomes due to increased cellular turnover as a result of primary myopathy [47]. Either of these factors alone or in combination will almost certainly lead to a decline in genome copy number per cell, leading to loss of efficacy over time. Third, early phase studies include subjects in low-dose cohorts and therefore would not receive a clinically effective dose [26,48]. This may impact on the need for re-exposure to the therapeutic vector. In order to ethically manage such studies in rare disease populations, it is appropriate to have a strategy in hand to remove the confounding effects of anti-capsid immunity and allow such subjects the chance to enter subsequent studies where a more effective dose is being proposed.

The preliminary results related to this single patient warrant a more in depth study of the mechanism of both B-cell and T-cell interactions following exposure to AAV capsids in humans. We propose future studies to test this clinical strategy in a prospective trial, thereby allowing for randomization in dose escalation studies or future re-administration.

Methods

This study was approved by the Institutional Review Board at the University of Florida and all procedures were performed in consideration of the Declaration of Helsinki. All patients gave their written informed consent before participating to this study. The study is registered at clinicaltrials.gov, identifier NCT00976352.

Outcome measures

Safety measures—Laboratory data and clinical evaluation confirmed safety of the procedure. Other safety laboratory studies included quantitative real-time polymerase chain reaction (PCR) to assess the presence of vector DNA in peripheral blood. Serum samples were assayed by enzyme linked immunosorbent assay (ELISA) for circulating antibodies to

the intact AAV1 capsid and hGAA protein [21,49]. Anti-AAV1 and anti-hGAA ASR and ELISPOT assay were performed as previously described on isolated peripheral blood mononuclear cells [50].

Enumeration of B- and T-lymphocyte subpopulations in peripheral blood was done by flow cytometry using monoclonal Abs to CD19 or CD20 for B cells and monoclonal Abs to CD3, CD4, and CD8 for T cells. Sirolimus trough levels, IgG, CD3, CD4, CD8, CD19, CD20, N-terminal pro-brain natriuretic peptide, creatine kinase, creatine kinase-MB, C-reactive protein, platelets, alkaline phosphatase, gamma-glutamyl transferase, aspartate aminotransferase, and alanine aminotransferase were measured at regular intervals.

Functional measures

MIP was evaluated using a one-way inspiratory occlusion. A one-way valve permitted exhalation, but prevented inspiratory airflow. A pressure transducer was used to record the negative pressure. The test was repeated three times and the most negative pressure was recorded.

GMFM-88 [51], which is an evaluative tool designed to measure change in the gross motor function of patients over time (i.e. lying/rolling, sitting, crawling/kneeling, standing and walking/running/jumping) was also tested. MIP and GMFM-88 were collected at baseline, day 90 and day 180.

Acknowledgements

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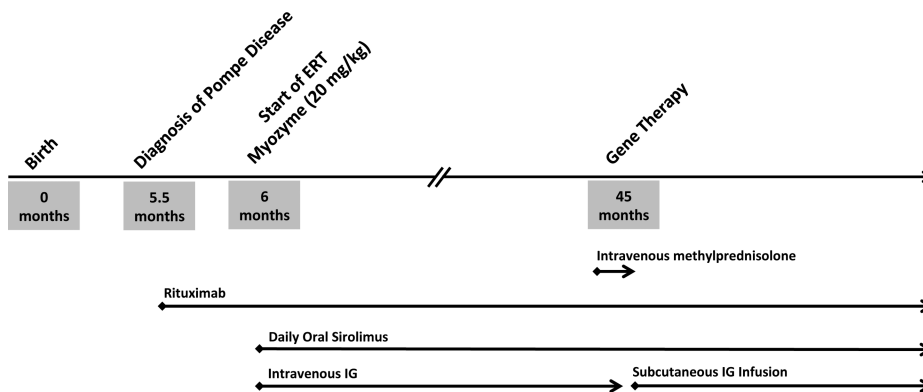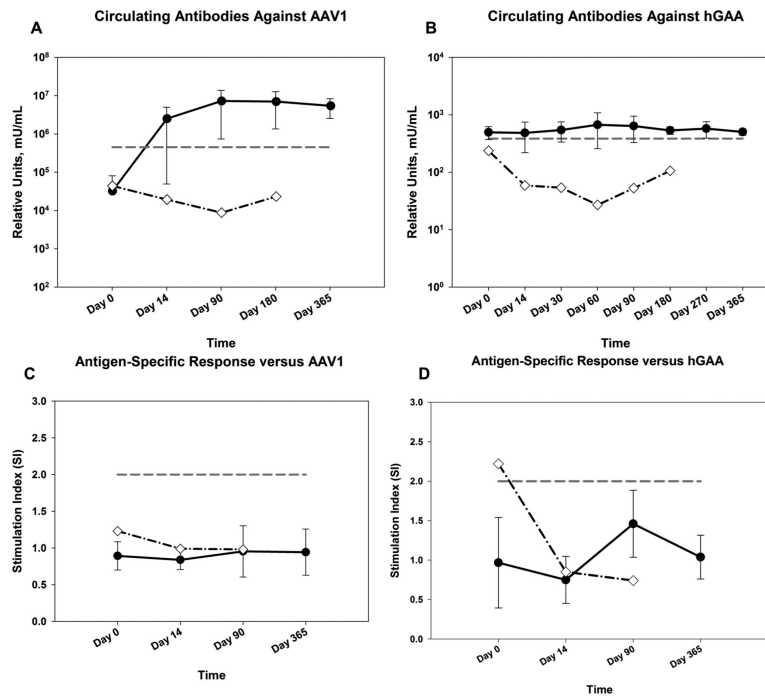
Figure 1.

Timeline: At 5.5 months of age the patient received an induction dose of Rituximab followed by daily oral Sirolimus. A monthly dose of IV immunoglobulin (IVIG) was given. The patient continued B-cell depletion regimen with Rituximab at 12 weeks intervals while receiving Sirolimus daily. At 6 months of age, after initiation of B cell depletion, the patient started on ERT infusion at a dose of 20 mg/kg every 7-10 days.. Peri-procedure methylprednisolone was given followed by AAV1-GAA intramuscular gene transfer to the diaphragm at 45 months of age.

Figure 2.
A 3.5 year-old Pompe disease subject (solid lines, diamonds) received weekly administration of 375 mg/m^2 of rituximab along with 10 mg/kg of methylprednisolone for 3 weeks prior to dosing with 5×10^{12} vg/kg of rAAV1-CMV-hGAA. This patient then received daily oral administration of 0.06-1 mg/m^2 of Sirolimus through the study and continued B-cell depletion with Rituximab every 12 weeks. Five Pompe subjects (dash- dot lines, circles), age 2.5 to 15 years, received $1-5 \times 10^{12}$ vg/kg of rAAV1-CMV-GAA served as AAV-treated, controls for B-cell depletion. The light horizontal lines represent +2 SD. population level. (a) Circulating antibodies against AAV1. (b) Circulating antibodies against hGAA. (c) Antigen specific response assay versus AAV1. (d) Antigen specific response assay versus hGAA .

Table 1

Safety data

	Day-30	Day -1	Day 1	Day 3	Day 14	Day 90	Day 180	Day 270
IgG (mg/dL)	674	-	-	-	-	994	-	1209
IgA (mg/dL)	<5	-	-	-	-	<5	-	<5
WBC (thou/cu mm)	15.3	8.4	5.5	10.5	7.5	12.3	5.6	8.1
Platelet (thou/cu mm)	481	403	385	491	389	374	304	324
Neutrophil (%)	61	16.6	24	23.8	29	69.7	29	48.1
Lymphocyte (%)	12	58.5	63	60.5	49.2	19	50.9	39.8
Monocyte (%)	5	6.6	9	10.2	8	7.7	13	7
Eosinophil (%)	1	16.2	3	3	10.1	2.3	4.2	2.4
Basophil (%)	0	0.4	0	0.6	0.7	0.2	0.5	0.4
RBC (mill/cu mm)	4.42	4.61	4.33	4.75	4.54	5.17	5.12	5.2
AST	366	484	445	438	469	496	575	436
ALT	246	306	287	316	239	277	426	292
CD20%	0	-	-	-	-	0	-	4
CD3%	80	-	-	-	-	86	-	88
NT-PRO-BNP	225	-	-	-	-	44	-	-
CK (U/L)	2549	1967	1518	1170	2433	2858	2613	-
CKMB (ng/mL)	22.7	-	-	-	-	22.8	-	-

Table 2

Functional data

	Day -1	Day 90	Day 180
Maximal Inspiratory Pressure (MIP) (cmH20)	**58.75**	**58.75**	**62.45**
Gross Motor Function Measure (GMFM - 88)			
Lying & rolling (% score)	23.53	33.33	23.53
Sitting (% score)	28.33	33.33	21.66
Crawling & Kneeling (% score)	0	0	0
Standing (% score)	0	0	0
Walking, Running, & Jumping (% score)	0	0	0
Total (% score)	**10.37**	**13.32**	**9.04**