Title: Disease-modifying immune-modulatory effects of the N-163 strain of Aureobasidium pullulans-produced 1,3-1,6 Beta glucans in young boys with Duchenne muscular dystrophy: Results of an open-label, prospective, randomized, comparative clinical study

Running Title: N-163 beta glucan as disease modifying agent in DMD

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Conflicts of interest/Competing interests: Author Samuel Abraham is a shareholder in GN Corporation, Japan which in turn is a shareholder in the manufacturing company of the Beta Glucans described in the study.

Ethics Approval:

The study was registered in Clinical trials registry of India, CTRI/2021/05/033346. Registered on 5th May, 2021. The study was approved by the Institutional Ethics Committee (IEC) of Saravana Multispeciality Hospital, India on 12th April, 2021.

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Abstract

Background: Duchenne muscular dystrophy (DMD) is an inherited neuromuscular disorder causing progressive muscle weakness and premature death. Steroids remain the mainstream approach for supportive care but have side effects; other targeted therapies and gene therapies are also being developed. As there is limited evidence on the use of disease-modifying nutritional supplement adjuncts in DMD, this pilot trial is to evaluate the effects of supplementation of Aureobasidium pullulans-derived 1,3-1,-6 beta glucan from the N-163 strain in young patients with DMD.

Methods:

Twenty-seven patients with Duchenne muscular dystrophy (DMD)—nine in the control arm (undergoing conventional therapies)—participated. The patients were divided into groups: those not administered steroids (Steroid -ve) (n = 5), those administered steroids (Steroid +ve) (n = 4), and 18 in the treatment arm (N-163 beta glucan supplement along with conventional therapies; N-163 Steroid –ve and N-163 Steroid +ve); they participated in the study for 45 days. Assessments of muscle function, disease status, and levels of IL-6, IL-13, TGF-β, creatinine kinase (CK), titin, TNF-α, haptoglobin, and dystrophin in the blood and myoglobin in the urine were performed at baseline and at the end of the study.

Results:

IL-6 showed a significant decrease in the N-163 Steroid -ve group, from a baseline value of 7.2 ± 1.2 pg/ml to 2.7 ± 0.03 pg/ml. IL-13 decreased in both treatment groups—from 157.76 ± 148.68 pg/ml to 114.08 ± 81.5 pg/ml (N-163 Steroid -ve) and from 289.56 ± 232.88 pg/ml to 255.56 ± 214.13 pg/ml (N-163 Steroid +ve). TGF-β levels showed a significant decrease in the N-163 Steroid –ve group, from a baseline value of 3302 ± 1895 ng/ml to 1325.66 ± 517 ng/ml post intervention. Dystrophin levels increased by up to 32% in both Steroid +ve and -ve groups.

Medical research council (MRC) grading showed muscle strength improvement in 12 out of 18 patients (67%) in the treatment group and four out of nine (44%) subjects in the control group.

Conclusion:

Supplementation with the N-163 beta glucan food supplement produced disease-modifying beneficial effects: a significant decrease in inflammation and fibrosis markers, increase in dystrophin and improvement in muscle strength in DMD subjects over 45 days, thus making this a potential adjunct treatment for DMD after validation. A longer duration of follow-up and further research on the mechanism of action and commonalities with other diseases provoked by hyperactive inflammation and/or fibrosis may pave the way for their extended applications in other dystrophinopathies and neuroinflammatory diseases.

Trial registration: Clinical trials registry of India, CTRI/2021/05/033346. Registered on 5 May, 2021.

Introduction:

Duchenne muscular dystrophy (DMD) is a devastating X-linked neuromuscular disorder causing severe and progressive weakness of skeletal muscles, leading to loss of ambulation along with concomitant impairment of cardiac and respiratory muscles and early mortality. Mutations in the dystrophin gene, which cause total loss of the dystrophin protein [2], remain the major underlying mechanism. Loss of dystrophin leads to damage of the myofibres' plasma membranes and distorts the structural stability of the plasma, leading to weakness in the myofibres. The weakened myofibres cannot withstand the contraction and relaxation cycles occurring during muscle function. The damage to the membrane releases the cytoplasmic contents, triggering the immune system and causing further muscle fibre damage, weakness and ultimately death [3]. A chronic proinflammatory state ensues, with neutrophil infiltration and macrophages' phagocytosis of the degenerated tissue [3], preventing repair of the muscle damage, which otherwise occurs in a highly orchestrated manner for faster repair in other physiological conditions. The muscle is relatively immunologically privileged, with a low capacity to generate localized immune responses and thus having low rates of abscess and granuloma formation [3]. Therefore, it becomes essential to modulate the inflammation and immunity to resolve the chronic inflammatory state in therapeutic approaches to DMD. Steroid therapy is the most commonly employed immuno-modulatory treatment approach. However, side effects include weight gain, weak bones, high blood pressure and behaviour changes in addition to muscle weakness and atrophy in the long term [4,5]. Thus, there arises the need to develop strategies that will assist in immunomodulation with lesser side effects. Nutritional supplements are a potential option. Beside beta glucans yielding locomotor improvement in zebrafish models of DMD [6], a 1-3,1-6 beta glucan from the N-163 strain of the black yeast Aureobasidium pullulans has been reported to mitigate inflammation, evident by decreases in anti-inflammatory markers such as CD11b, serum ferritin, galectin-3 and fibrinogen. It also

produces beneficial immuno-modulation via a decrease in the neutrophil-to-lymphocyte ratio (NLR) and an increase in the lymphocyte-to-CRP ratio (LCR) and leukocyte-to-CRP ratio (LeCR) in human healthy volunteers [7]. Mitigation of lipotoxicity-associated inflammatory cascades in a mouse study has also been reported [8]. Another study done in an animal model of non-alcoholic steatohepatitis (NASH) showed a decrease in liver inflammation and accumulation of F4/80+ cells (macrophages associated with inflammation) [9] in the liver. The present pilot study is to evaluate the immunomodulatory efficacy of the N-163 strain of A. pullulans-produced beta 1-3,1-,6 glucan in comparison with a conventional therapeutic regimen in patients with DMD.

Methods:

This trial was an investigator-initiated, single-centre, randomized, open-label, prospective, comparative, two-arm clinical study of patients with DMD. The study was conducted over 45 days. The two treatment arms included

Treatment arm I, control arm: Conventional treatment regimen comprising standard routine physiotherapy for joint mobility along with medications, viz., T. calcium and vit. D 1000 with or without T. deflocort (steroid) 6mg to 24 mg.

Treatment arm II, intervention: One sachet of N-163 beta glucan (16 mg gel) once daily along with conventional treatment.

Inclusion criteria: Male subjects with molecular diagnosis of DMD aged 6-18 years who were willing to participate in the study with written informed consent.

Exclusion criteria: Patients with a previous (within the past 1 month) or concomitant participation in any other therapeutic trial; a known or suspected malignancy; any other chronic disease or clinically relevant limitation of renal, liver or heart function according to the discretion of the investigator.

Investigations:

The following tests were carried out after written consent was obtained from the study subjects.

At baseline and at the end of the study (after 45 days):

- Background survey: gender, date of birth, age, habits, current medical history, medication, treatment, allergies (to drugs and food), regular use of food for specified health uses, functional foods, health foods, intake of foods rich in β-glucan foods containing beta-glucan and intake of immunity-boosting foods
- Medical history and physical measurements: height, weight, BMI, temperature
- Physiological examination: systolic blood pressure, diastolic blood pressure, pulse rate
- ECG
- Muscle strength test using MRC grading [10]
- Six-minute walk test (6MWT) [11]
- North Star Ambulatory Assessment (NSAA) [12]
- Blood sampling and investigations for the levels of IL-6, IL-13, TGF-β, creatinine kinase (CK), titin, haptoglobin, TNF-α, dystrophin, cystatin in the blood and myoglobin in the urine
- Subjects were contacted every week for drug compliance and recording of adverse effects, if any

Study subjects = 28

The study was designed as an exploratory study, so there were two intervention conditions: one control and one test group. As the minimum number of participants required for statistical comparisons within and between intervention conditions is four per intervention condition, a

total of 28 target study participants (10 in treatment arm I [control] group and 18 in treatment arm II [N-163]) were used.

Selection of study subjects

Study investigators and other investigators included study subjects who had consented to participate in the study, met the selection criteria and not the exclusion criteria, and who were judged to have no problem participating in the study.

Allocation of study subjects

The person in charge of the allocation, as specified in the study protocol, allocated the study subjects to the two groups by simple randomization.

Primary outcome:

Observation of changes in the levels of IL-6 and myoglobin urea from the baseline.

Secondary outcome:

- Observation of changes in the levels of IL-6, IL-13, TGF-β, CK, titin, dystrophin, haptoglobin, cystatin C, and TNF-α in the serum and urine myoglobin levels measured by ELISA.
- Monitoring for adverse effects

Statistical analysis:

All data were analysed using Excel statistics package analysis software (Microsoft Office Excel®); Student's t-tests and ANOVA were used. When there was a significant main effect, post hoc pairwise comparisons were performed, and p-values < 0.05 were considered significant.

Results:

Twenty-eight patients were screened and 27 were randomized to control (n = 9) and treatment (n = 18). One patient was disqualified due to misrepresentation of diagnosis. The CONSORT flow diagram of the trial is shown in Figure 1.

Demographics are shown in Table 1. The mean ± SD age for the total study population was 11.18 ± 3.86 years (range 5-19 years) and was similar across the groups. The mean ± SD body weight was 35.59 ± 15.5 kgs (range = 10 to 65 kgs).

The distribution of patients was as follows:

Group I: Control group (n = 9);

A. Steroids not administered (n = 5) (Steroid -ve)
B. Steroids administered (n = 4) (Steroid +ve)

Group II: Treatment (N-163) group (n = 18);

A. Steroids not administered (n = 9) (Steroid -ve)
C. Steroids administered (n = 9) (Steroid +ve)

No adverse events were reported. No clinically significant changes from baseline data were observed on physical examination or in vital signs—temperature, blood pressure, oxygen saturation, pulse rate or ECG (data not shown).

Biomarker levels:

Levels are expressed as mean ± SD. IL-6 showed the highest decrease in the N-163 Steroid -ve group, from a baseline value of 7.2 ± 1.2 pg/ml to 2.7 ± 0.03 ng/ ml post intervention, but the difference was not significant (p-value = 0.16) (Figure 2).

IL-13 increased in both control groups—from 300.4 ± 114.5 pg/ml at baseline to 550.732 ± 107.95 pg/ml post-intervention in the Steroid -ve group and from 142 ±112.82 pg/ml at baseline to 263.5 ± 99.38 pg/ml post-intervention in the Steroid +ve group. It decreased in both the treatment groups—from 157.76 ± 148.68 pg/ml at baseline to 114.08 ± 81.5 pg/ml post-intervention and from 289.56 ± 232.88 pg/ml at baseline to 255.56 ± 214.13 pg/ml post-intervention. The difference was statistically significant (p-value = 0.004) (Figure 3).

TGF-β levels showed a significant decrease in the N-163 Steroid -ve group, from a baseline value of 3302 ± 1895 ng/ml to 1325.66 ± 517 ng/ml post intervention, which was significantly lower than all the other groups (p-value = 0.0001) (Figure 4).

Dystrophin levels showed a significant increase in the N-163 Steroid -ve group, from a baseline value of 3.01 ± 1.58 ng/ml to 4.01 ± 1.44ng/ml post intervention, and the N-163 Steroid +ve group went from a baseline value of 3.15 ± 2.43 ng/ml to 3.78 ± 2.17 ng/ml post intervention, which was significantly higher than the control groups (p-value = 0.0009) (Figure 2). The N-163 Steroid -ve group showed higher dystrophin expression than the N-163 Steroid +ve group, but the difference was not significant (p-value = 0.11). The percentage increase in dystrophin levels in the treatment group was up to 32.8%.

Haptoglobin did not show much difference pre or post intervention in the treatment groups, but it was marginally increased in the control group (Figure 6A). CK increased in the treatment groups (Figure 6B). Urine myoglobin increased in the N-163 Steroid +ve group but decreased in all the other groups (Figure 6C).

Titin and cystatin C decreased in the N-163 Steroid +ve group and the control Steroid +ve group, but the difference was not significant (Figure 7A.B). TNF-α decreased in all the groups except the N-163 Steroid -ve group.

The 6MWT and NSAA did not show any significant differences between the groups (Figure 8 A, B). The MRC grading showed improvement in 12 out of 18 patients (67%) in the treatment group and only four out of nine (44%) subjects in the control group (Table 2).

Discussion:

Current interventions for DMD, such as corticosteroids and rehabilitative care, help to prolong survival up to the third or the fourth decade of life. Corticosteroids remain the mainstream supportive approach to slow inflammation and the associated decline in muscle strength and function [4]. However, steroids have their own adverse effects, and their prescription is based on risk versus benefit to that specific patient and tolerance to the medication. Exon-skipping gene therapy and cell-based strategies to replace the mutant DMD gene are in development, but the desired outcome has not yet been achieved. In the meantime, nutraceuticals can be considered potential strategies for immune modulation and alleviating inflammation, as they are safer with lesser adverse effects [4]. Improvement of the locomotor performances and mitochondrial respiration by 1,3-1,6 beta-glucans in zebra fish model of muscular dystrophy [6] has already been reported.

In the current study, we focussed on a 1-3,1-6 beta glucan from the N-163 strain of the black yeast A. pullulans that has been reported to mitigate inflammation, evidenced by a decrease in anti-inflammatory markers and production of beneficial immuno-modulation [6-8]. The safety profile of N-163 beta glucan has been confirmed by the results.

Anti-inflammatory and anti-fibrotic outcomes: Circulating IL-6 is chronically elevated in individuals with DMD [13], which has been reported to contribute to DMD-associated cognitive dysfunction. IL-6 blockades have been advocated as a therapeutic approach for DMD [14]. In the present study, IL-6 showed highest decrease in the N-163 Steroid -ve group (Figure 2). While IL-6 is an acute inflammatory biomarker [14], IL-13 is a pro-fibrotic

biomarker [15] and was significantly decreased (Figure 3). Together with the TGF-β pathway, it is a major proinflammatory and pro-fibrotic cytokine responsible for the chronic inflammatory response leading to replacement of the muscle by scar tissue or fibrosis, resulting in muscle weakness and loss of muscle function [16]. TGF-β levels also showed a significant decrease in the N-163 Steroid -ve group (Figure 4). Dystrophin restoration of 20% expression [17,18] is considered the point of efficacy for a DMD therapy [19] and was found to increase by 32.8% in both the treatment groups (Figure 5) of the present study from baseline. This establishes N-163 beta glucan as an efficient agent for DMD. This dystrophin increase could be attributed to the immune modulation proven through control of anti-inflammatory and anti-fibrotic markers (IL-6, IL-13 and TGF-β).

Other biochemical markers of relevance: While haptoglobin and urine myoglobin did not show significant differences, the increase in urine myoglobin in the N-163 Steroid +ve group deserves more analysis concerning the underlying mechanism. Greater activity among steroid-treated individuals may place their dystrophin-deficient muscles under greater mechanical stress, predisposing them to further muscle fibre damage and consequent myoglobinuria [20]. While titin and cystatin C decreased in the N-163 steroid +ve group and in the control Steroid +ve group, there was an increase in CK, which is paradoxical, as reports suggest that titin concentration correlates significantly with serum CK concentration [21].

Muscle strength evaluation: There were three evaluations to assess muscle strength and tone, done in a blinded manner by the same physiotherapist at baseline and post intervention. Though the 6MWT and NSAA did not show any significant differences between the groups, MRC grading showed improvement of muscle strength in 67% of the subjects in the treatment group compared to 44% subjects in the control group, which is significant. The limitation of this being a 45-day study is relevant to the muscle-strength and functional evaluations, mandating the need for a longer study and follow-up duration. However, though small, the improvement in

MRC grading at 45 days could be again attributed to the immune modulation effects of this disease-modifying supplement. The study shows proof of concept that DMD could be tackled by the N-163 beta glucan from three aspects: decrease in inflammation shown by decreased IL-6 and TNF-α, decrease in fibrosis evident by decreased TGF-β and IL-13 and, more importantly, restoration of dystrophin evident from a 32.8% increase in dystrophin levels. These effects hold regardless of the use or non-use of steroids, which is important, as this safety-proven food supplement can help DMD patients regardless of steroid status.

Chronic inflammation being common to pathogenesis of all muscular dystrophies, immunomodulatory treatment may benefit patients with diverse types of muscular dystrophy [22]. Further, modulating the inflammatory response and inducing immunological tolerance to de novo dystrophin expression is critical to the success of dystrophin-replacement therapies [23]. The need to evaluate the muscles involved in respiratory function and myocardium should be mentioned here, as they are the cause of mortality in most of the DMD patients [1]. Though other dystrophinopathies, such as limb girdle muscular dystrophy (LGMD), do not involve respiratory or cardiac muscles, inflammatory overactivity is the common pathophysiology among types of muscular dystrophy [22]. Once proven efficacious for DMD, extending the beneficial application of the N-163 beta glucans to other dystrophinopathies such as LGMD can be considered.

DMD is a rare genetic disease with a maximum life expectancy of up to fourth decade, with the majority of victims dying in their late twenties to thirties. The average lifespan at birth, which was 20+ years for those born in or before 1970, has gradually increased by 10~15 years for those born and diagnosed with DMD in the 1980s and 1990s. This increase is attributed to better or early ventilatory assistance, steroid usage and cardiac care [24,25], which are only supportive interventions. With the gene therapies approved recently, there is a hope of

additional progress and increase in lifespan [26]. Though these gene therapies (such as exon skipping) address the root cause by splicing out selected exons from the pre-mRNA at or next to the mutation site, generating a translatable transcript from the mutant dystrophin gene leading to dystrophin expression [26, 27], they are still marred by challenges such as delivery of gene-editing components throughout the musculature and mitigation of possible immune responses [28]. The current need, therefore, is to modulate the immune system and control the inflammation and ensuing fibrosis to delay the progression of the disease. The earlier usage of steroids in a regular manner was later changed to intermittent usage [29] with regimens varying between institutes; now, newer steroids with lesser adverse effects are in various stages of progress towards clinical applications [30]. In this background, the safety of this N-163-produced beta glucan food supplement without adverse reactions is to be considered an indispensable value addition. Targeting the inflammation component (the criteria for selecting this supplement for this study) having yielded beneficial outcomes, additional studies on this characteristic could be of value to possibly extending their application for other neuroinflammatory diseases, such as multiple sclerosis. At this point, it is essential to mention the gut microbiome for two reasons; one being the association of the microbiome with the severity of neuroinflammatory conditions such as multiple sclerosis [31], and another being the fact that beta glucans have been reported to yield beneficial reconstitution of the gut microbiome in earlier studies [32] in children with autism spectrum disorder, a neurodevelopmental disease. For both multiple sclerosis and DMD, steroids to suppress inflammation are common, but associated implications for gut microbiota in DMD have not been reported often and are worthy of future study.

The limitations of the study include uneven distribution of subjects and short follow-up (only 45 days); improvements in muscle function over the course of the study showed variability that may have been due to the level of sensitivity to change of functional assessments during the

365 disease progression in the age group. Among the 27 subjects, two-thirds were ambulatory and
366 the remaining non-ambulatory; the evaluation criteria differences must be kept in mind, which
367 may show equivalent quantification among all DMD patients at different stages of disease
368 severity when non-invasive myograms to measure the individual muscles accurately could be
369 undertaken. Further, consumption of steroids vs those who did not consume them or those who
370 had stopped steroids after an initial duration of consumption, as well as regimen variation, are
371 to be considered while interpreting the outcomes. All these aspects mandate the need for larger
372 randomized clinical trials of longer duration to validate this supplement as a treatment.

373 **Conclusion:**

374 N-163 beta glucan with and without steroids helped decrease IL-6, TGF-β and IL-13 and
375 increase dystrophin levels along with improvement of muscle strength in subjects with DMD
376 in this clinical study. Thus, N-163 beta glucan is a safe and effective potential therapeutic
377 disease-modifying adjunct for patients with DMD. While the benefits documented may help
378 slow the rate of progression of this devastating disease, confirmation by longer and larger
379 studies will help establish this agent for routine clinical application as a disease-modifying
380 agent with the potential to help prolong the lifespan of DMD patients. After such validation,
381 extending its application to other dystrophinopathies such as LGMD could be considered, and
382 further in-depth research on gut microbiomes and their implications in neuroinflammatory
383 diseases are likely to shed light on the mechanism of action, leading to additional beneficial
384 applications.

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References:

1. Theadom A, Rodrigues M, Roxburgh R, Balalla S, Higgins C, Bhattacharjee R, Jones K, Krishnamurthi R, Feigin V. Prevalence of muscular dystrophies: a systematic literature review. Neuroepidemiology. 2014;43(3-4):259-68.
2. Nagy S, Hafner P, Schmidt S, Rubino-Nacht D, Schädelin S, Bieri O, Fischer D. Tamoxifen in Duchenne muscular dystrophy (TAMDMD): study protocol for a multicenter, randomized, placebo-controlled, double-blind phase 3 trial. Trials. 2019;20(1):637.
3. Rosenberg AS, Puig M, Nagaraju K, Hoffman EP, Villalta SA, Rao VA, Wakefield LM, Woodcock J. Immune-mediated pathology in Duchenne muscular dystrophy. Sci Transl Med. 2015;7(299):299rv4.
4. Manzur AY, Kuntzer T, Pike M, Swan A. Glucocorticoid corticosteroids for Duchenne muscular dystrophy. Cochrane Database Syst Rev. 2004;(2):CD003725.
5. National Institute of Arthritis and Musculoskeletal and Skin Diseases. Spotlight on Research: Optimizing Steroid Treatment for Duchenne Muscular Dystrophy https://www.niams.nih.gov/newsroom/spotlight-on-research/optimizing-steroid-treatment-duchenne-muscular-dystrophy (Accessed: December 13, 2021).
6. Licitra R, Marchese M, Brogi L, Fronte B, Pitto L, Santorelli FM. Nutraceutical Screening in a Zebrafish Model of Muscular Dystrophy: Gingerol as a Possible Food Aid. Nutrients. 2021;13(3):998.
7. Ikewaki N, Sonoda T, Kurosawa G, Iwasaki M, Dedeepiya VD, Senthilkumar R, Preethy S, Abraham SJK. Immune and metabolic beneficial effects of Beta 1,3-1,6 glucans produced by two novel strains of Aureobasidium pullulans in healthy middle-aged Japanese men: An exploratory study. medRxiv 2021.08.05.21261640; doi: 10.1101/2021.08.05.21261640

8. Ikewaki N, Onaka T, Ikeue Y, Nagataki M, Kurosawa G, Dedeepiya VD, Rajmohan M, Vaddi S, Senthilkumar R, Preethy S, Abraham SJK. Beneficial effects of the AFO-202 and N-163 strains of Aureobasidium pullulans produced 1,3-1,6 beta glucans on non-esterified fatty acid levels in obese diabetic KKAy mice: A comparative study.bioRxiv 2021.07.22.453362; doi: 10.1101/2021.07.22.453362
9. Ikewaki N, Kurosawa G, Iwasaki M, Preethy S, Dedeepiya VD, Vaddi S, Senthilkumar R, Levy GA, Abraham SJK. Hepatoprotective effects of Aureobasidium pullulans derived Beta 1,3-1,6 biological response modifier glucans in a STAM- animal model of non-alcoholic steatohepatitis. bioRxiv 2021.07.08.451700; doi: 10.1101/2021.07.08.451700
10. MRC Muscle Scale. https://mrc.ukri.org/research/facilities-and-resources-for-researchers/mrc-scales/mrc-muscle-scale/ (Accessed: December 13, 2021).
11. McDonald CM, Henricson EK, Han JJ, Abresch RT, Nicorici A, Elfring GL, Atkinson L, Reha A, Hirawat S, Miller LL. The 6-minute walk test as a new outcome measure in Duchenne muscular dystrophy. Muscle Nerve. 2010;41(4):500-10
12. Scott E, Eagle M, Mayhew A, Freeman J, Main M, Sheehan J, Manzur A, Muntoni F; North Star Clinical Network for Paediatric Neuromuscular Disease. Development of a functional assessment scale for ambulatory boys with Duchenne muscular dystrophy. Physiother Res Int. 2012;17(2):101-9. doi: 10.1002/pri.520.
13. Stephenson KA, Rae MG, O'Malley D. Interleukin-6: A neuro-active cytokine contributing to cognitive impairment in Duchenne muscular dystrophy? Cytokine. 2020;133:155134.
14. Mammen AL, Sartorelli V. IL-6 Blockade as a Therapeutic Approach for Duchenne Muscular Dystrophy. EBioMedicine. 2015;2(4):274-5.

15. Mann CJ, Perdiguero E, Kharraz Y, Aguilar S, Pessina P, Serrano AL, Muñoz-Cánoves P. Aberrant repair and fibrosis development in skeletal muscle. Skelet Muscle. 2011;1(1):21.

16. Ceco E, McNally EM. Modifying muscular dystrophy through transforming growth factor-β. FEBS J. 2013;280(17):4198-209.

17. Shimizu-Motohashi Y, Komaki H, Motohashi N, Takeda S, Yokota T, Aoki Y. Restoring Dystrophin Expression in Duchenne Muscular Dystrophy: Current Status of Therapeutic Approaches. J Pers Med. 2019;9(1):1.

18. Mendell JR, Rodino-Klapac L, Sahenk Z, Malik V, Kaspar BK, Walker CM, Clark KR. Gene therapy for muscular dystrophy: lessons learned and path forward. Neurosci Lett. 2012;527(2):90-9.

19. Wells DJ. What is the level of dystrophin expression required for effective therapy of Duchenne muscular dystrophy? J Muscle Res Cell Motil. 2019 ;40(2):141-150.

20. Awano H, Matsumoto M, Nagai M, Shirakawa T, Maruyama N, Iijima K, Nabeshima YI, Matsuo M. Diagnostic and clinical significance of the titin fragment in urine of Duchenne muscular dystrophy patients. Clin Chim Acta. 2018;476:111-116.

21. Garrood P, Eagle M, Jardine PE, Bushby K, Straub V. Myoglobinuria in boys with Duchenne muscular dystrophy on corticosteroid therapy. Neuromuscul Disord. 2008 ;18(1):71-3.

22. Raimondo TM, Mooney DJ. Anti-inflammatory nanoparticles significantly improve muscle function in a murine model of advanced muscular dystrophy. Sci Adv. 2021;7(26):eabh3693.

23. Thomas GD, Ye J, De Nardi C, Monopoli A, Ongini E, Victor RG. Treatment with a nitric oxide-donating NSAID alleviates functional muscle ischemia in the mouse model of Duchenne muscular dystrophy. PLoS One. 2012;7(11):e49350.

24. Ryder S, Leadley RM, Armstrong N, Westwood M, de Kock S, Butt T, Jain M, Kleijnen J. The burden, epidemiology, costs and treatment for Duchenne muscular dystrophy: an evidence review. Orphanet J Rare Dis. 2017;12(1):79

25. Kieny P, Chollet S, Delalande P, Le Fort M, Magot A, Pereon Y, Perrouin Verbe B. Evolution of life expectancy of patients with Duchenne muscular dystrophy at AFM Yolaine de Kepper centre between 1981 and 2011. Ann Phys Rehabil Med. 2013;56(6):443-54.

26. Duan D. Systemic AAV Micro-dystrophin Gene Therapy for Duchenne Muscular Dystrophy. Mol Ther. 2018;26(10):2337-2356.

27. Echevarría L, Aupy P, Goyenvalle A. Exon-skipping advances for Duchenne muscular dystrophy. Hum Mol Genet. 2018;27(R2):R163-R172.

28. Dzierlega K, Yokota T. Optimization of antisense-mediated exon skipping for Duchenne muscular dystrophy. Gene Ther. 2020;27(9):407-416. doi: 10.1038/s41434-020-0156-6.

29. Olson EN. Toward the correction of muscular dystrophy by gene editing. Proc Natl Acad Sci U S A. 2021;118(22):e2004840117.

30. Kourakis S, Timpani CA, Campelj DG, Hafner P, Gueven N, Fischer D, Rybalka E. Standard of care versus new-wave corticosteroids in the treatment of Duchenne muscular dystrophy: Can we do better? Orphanet J Rare Dis. 2021;16(1):117. doi: 10.1186/s13023-021-01758-9.

31. Boziki MK, Kesidou E, Theotokis P, Mentis AA, Karafoulidou E, Melnikov M, Sviridova A, Rogovski V, Boyko A, Grigoriadis N. Microbiome in Multiple Sclerosis; Where Are We, What We Know and Do Not Know. Brain Sci. 2020;10(4):234.

32. Raghavan K, Dedeepiya VD, Yamamoto N, Ikewaki N, Sonoda T, Kurosawa G, Iwasaki M, Kandaswamy R, Senthilkumar R, Preethy S, Abraham SJK. Beneficial

reconstitution of gut microbiota and control of alpha-synuclein and curli-amyloids-producing enterobacteria, by beta 1,3-1,6 glucans in a clinical pilot study of autism and potentials in neurodegenerative diseases. medRxiv 2021.10.26.21265505; doi: 10.1101/2021.10.26.21265505

509 **Tables and Figures**

510 Table 1: Demographics and baseline characteristics

	Subject	Age in years	Weight in kgs	Exon Deletion	Ambulatory/ Non-Ambulatory
Control Steroid -ve	1	7	12	10 & 11	Ambulatory
	2	10	27	46-55	Non-Ambulatory
	3	19	48		Ambulatory
	4	14	60	8 to 48	Non-Ambulatory
	5	15	55	10-11/Dup	Non-Ambulatory
Control Steroid +ve	1	19	65	44	Non-Ambulatory
	2	7	25	5 & 6	Ambulatory
	3	7	21	49-52	Ambulatory
	4	13	48	52	Ambulatory
N-163 Steroid -ve	1	10	28	48-50	Ambulatory
	2	4	10	45-50	Ambulatory
	3	13	38	45-50	Ambulatory
	4	10	45	60	Ambulatory
	5	7	39	10-11/ Dup	Non-Ambulatory
	6	8	21	49-52	Ambulatory
	7	13	39	43	Non-Ambulatory
	8	14	40		Non-Ambulatory
	9	7	22	44-57/Dup	Ambulatory
N-163 Steroid +ve	1	5	18	48-50	Non-Ambulatory
	2	14	59	17	Ambulatory
	3	15	40	60	Ambulatory
	4	10	20	48-52	Ambulatory
	5	10	36	48-50	Ambulatory
	6	14	58	18-29	Non-Ambulatory
	7	10	22	48-52	Ambulatory
	8	12	36	48-52	Non-Ambulatory
	9	15	29	07-Jun	Ambulatory

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515 Table 2: Medical research council (MRC) grading of muscle power in the study groups

Subject	Ambulatory/Non-ambulatory	Baseline	Post-intervention	Progression
Control group				
1	Ambulatory	151	165	Improved
2	Ambulatory	133	162	Improved
3	Non-ambulatory	70	80	Improved
4	Non-ambulatory	164	164	No change
5	Non-ambulatory	109	106	Worsened
6	Non-ambulatory	102	105	Improved
7	Non-ambulatory	117	115	Worsened
8	Non-ambulatory	110	109	No change
9	Non-ambulatory	105	102	Worsened
Treatment Group				
1	Ambulatory	134	133	Worsened
2	Ambulatory	146	163	Improved
3	Ambulatory	119	131	Improved
4	Ambulatory	127	136	Improved
5	Ambulatory	154	160	Improved
6	Ambulatory	158	168	Improved
7	Non-ambulatory	102	119	Improved
8	Non-ambulatory	116	117	Improved
9	Non-ambulatory	127	127	No change
10	Non-ambulatory	120	131	Improved
11	Non-ambulatory	96	107	Improved
12	Non-ambulatory	111	122	Improved
13	Non-ambulatory	117	113	Worsened
14	Non-ambulatory	108	108	No change
15	Non-ambulatory	103	107	Improved
16	Non-ambulatory	145	148	Improved
17	Non-ambulatory	93	119	Improved
18	Non-ambulatory	Test couldn't be performed		

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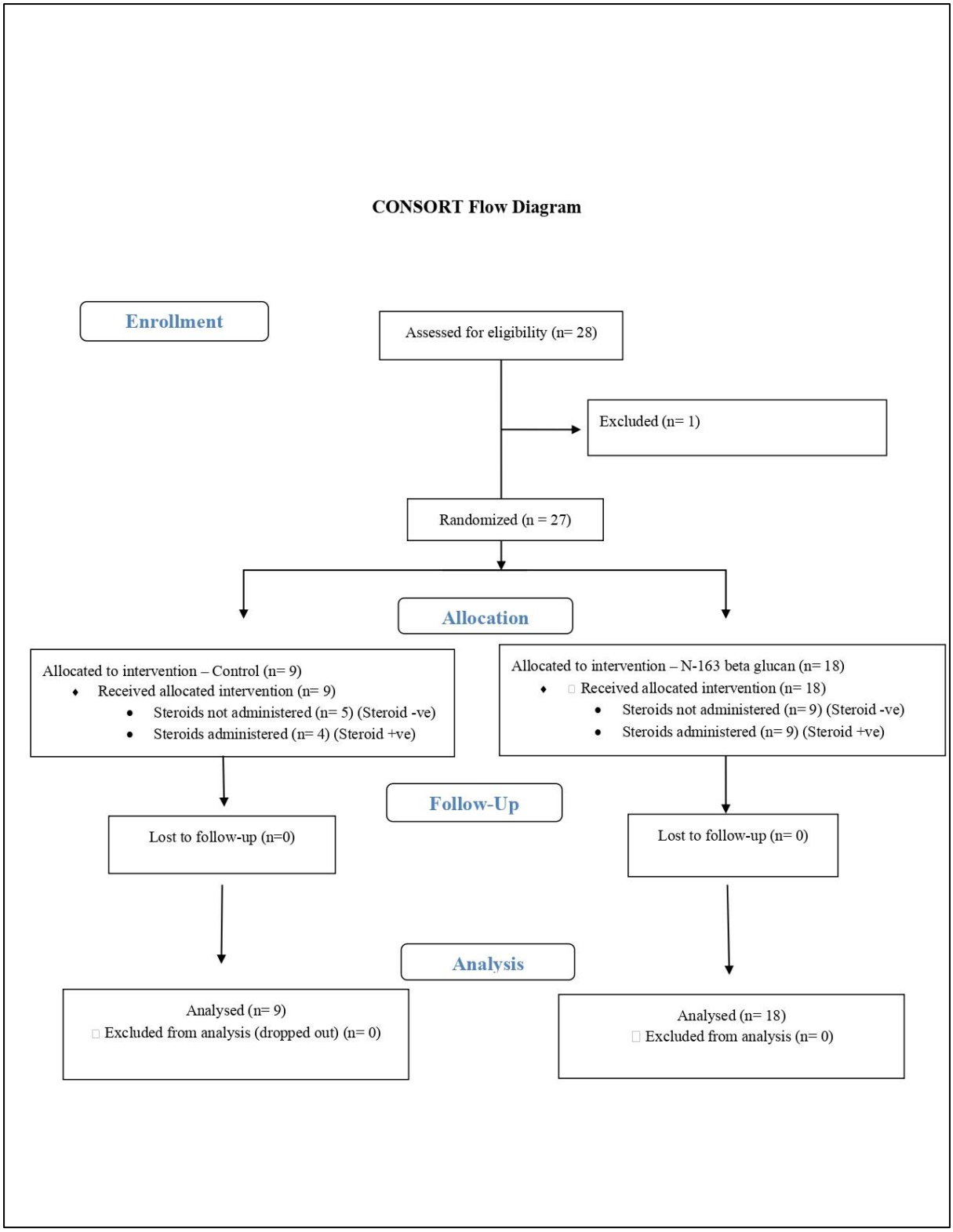
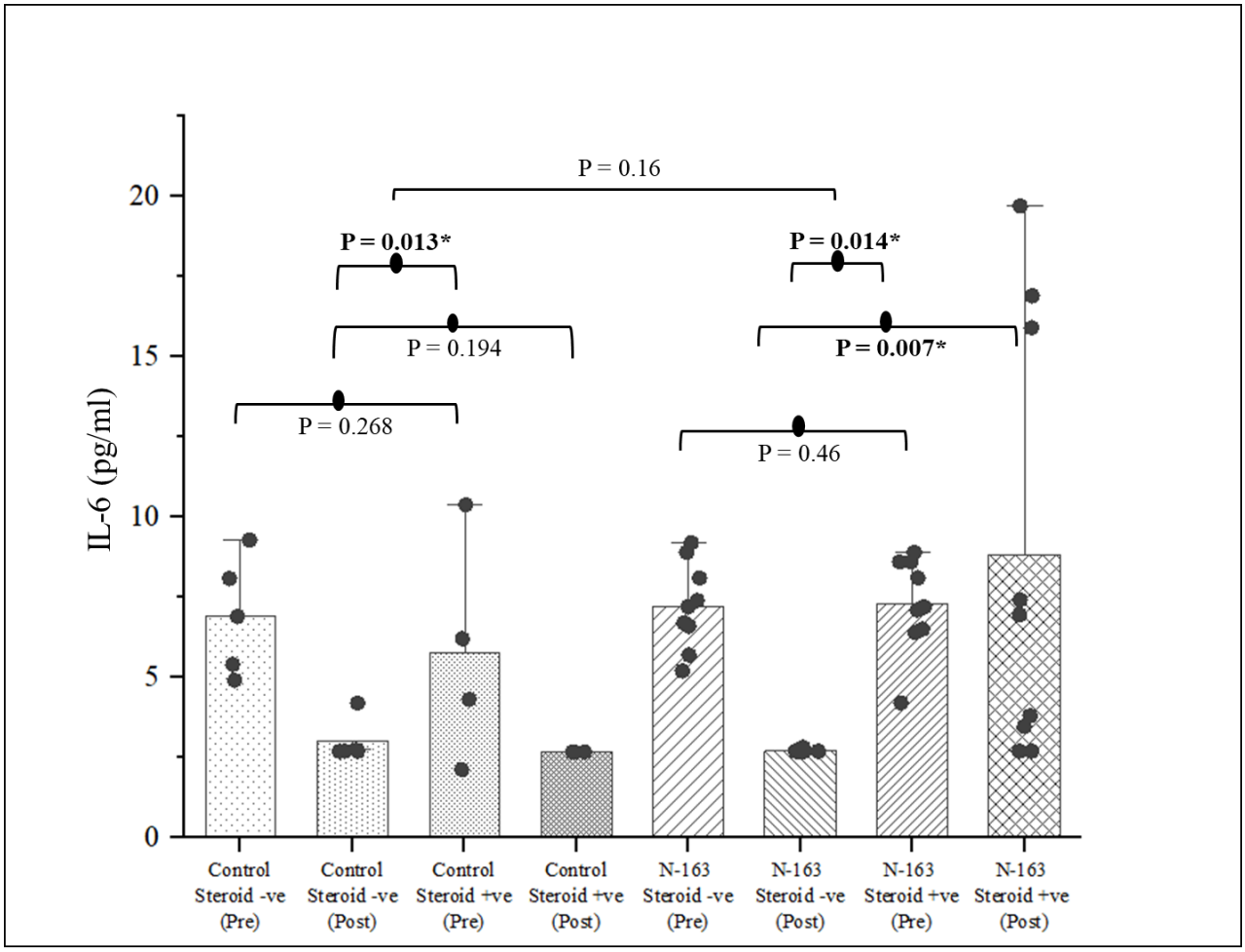


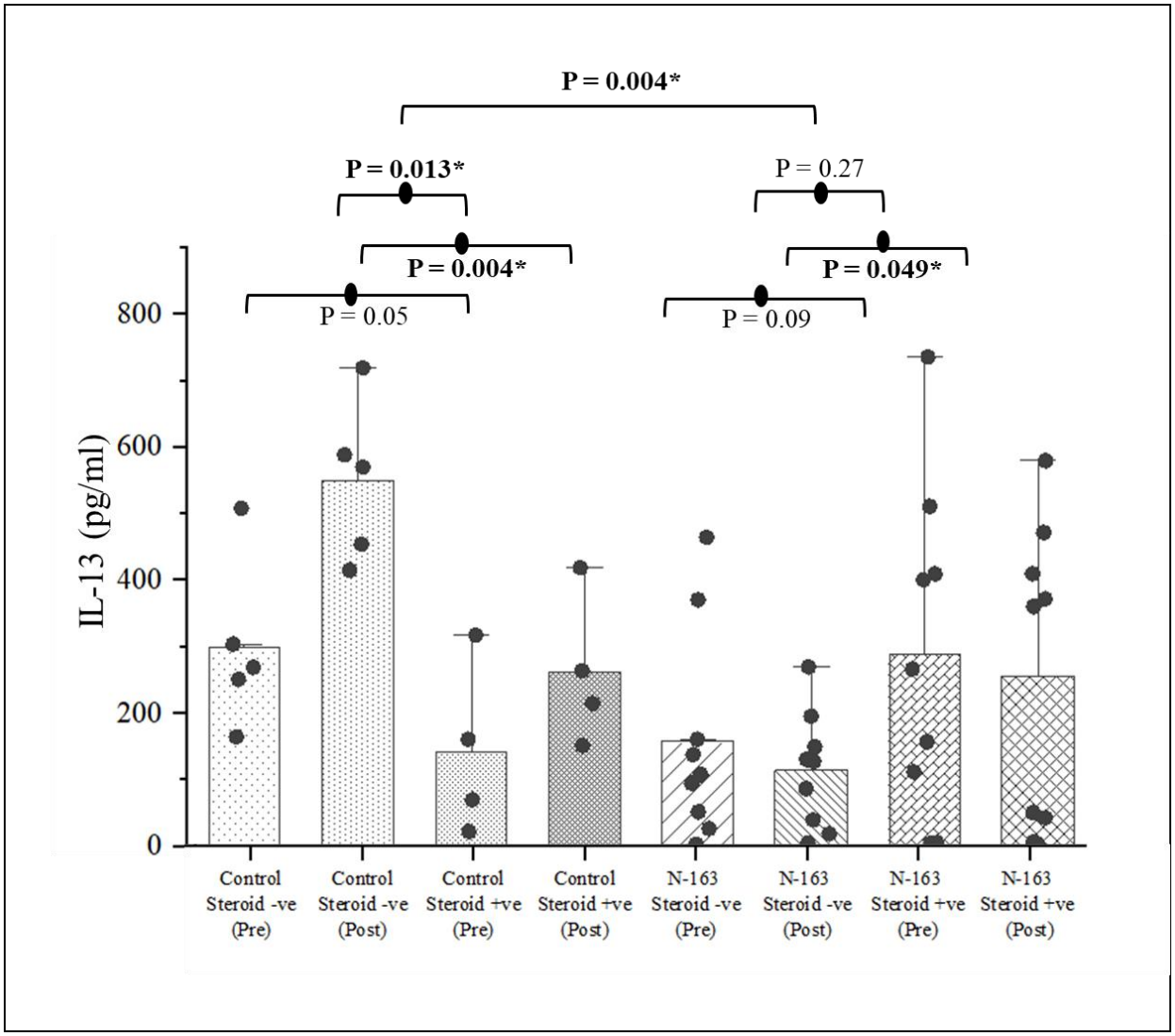
Figure 1: CONSORT flow diagram of the trial



520

521 Figure 2: IL-6 showed the most significant decrease in the N-163 Steroid -ve group compared

522 to other groups. (*p-value significance < 0.05)



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524 Figure 3: Levels of IL-13 levels showed statistically significant increase in control groups

525 and decrease in treatment groups (*p-value significance < 0.05)

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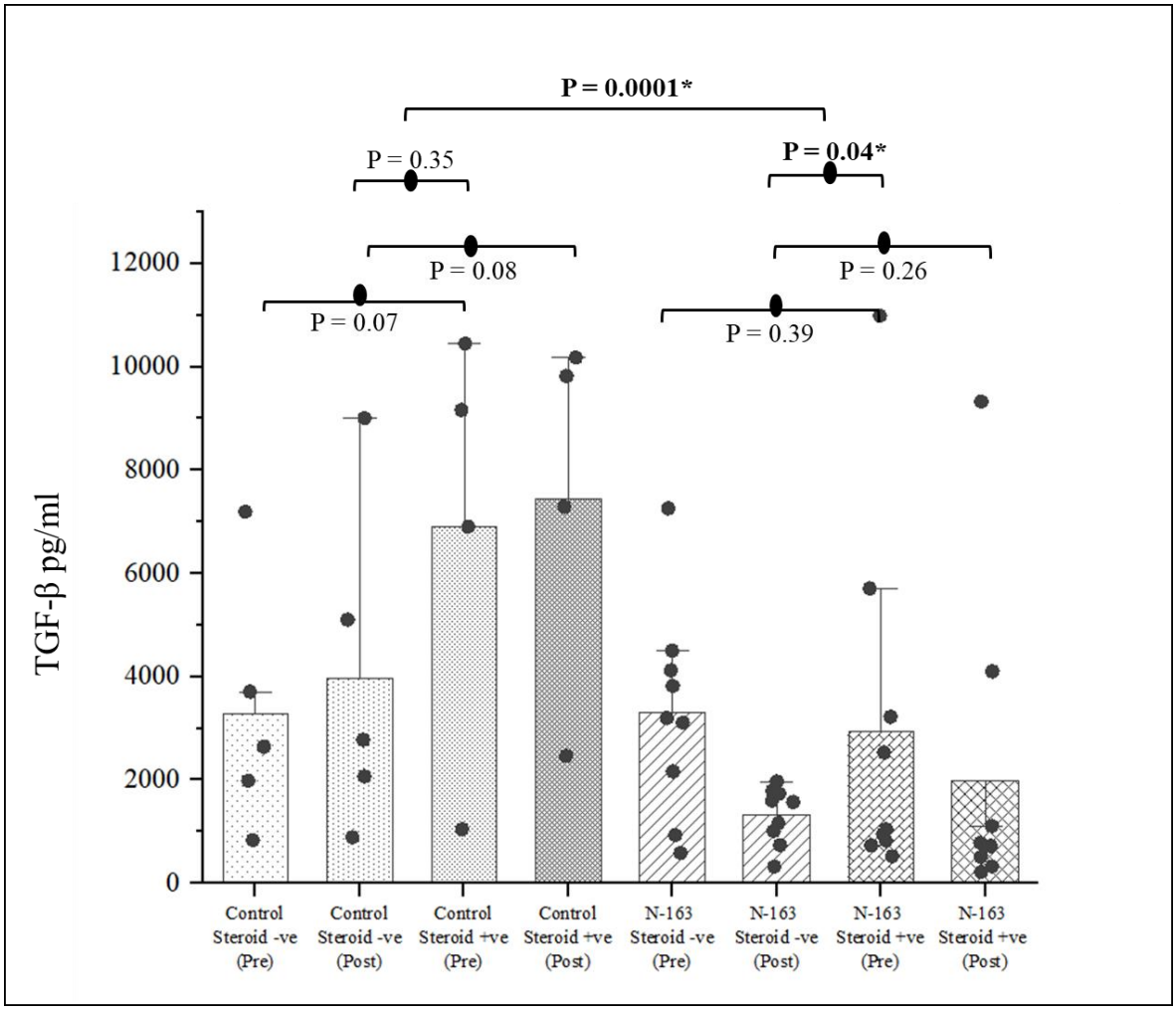

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528 Figure 4: Levels of TGF-β showed significant decrease in the N-163 Steroid -ve group

529 compared to other groups (*p-value significance < 0.05)

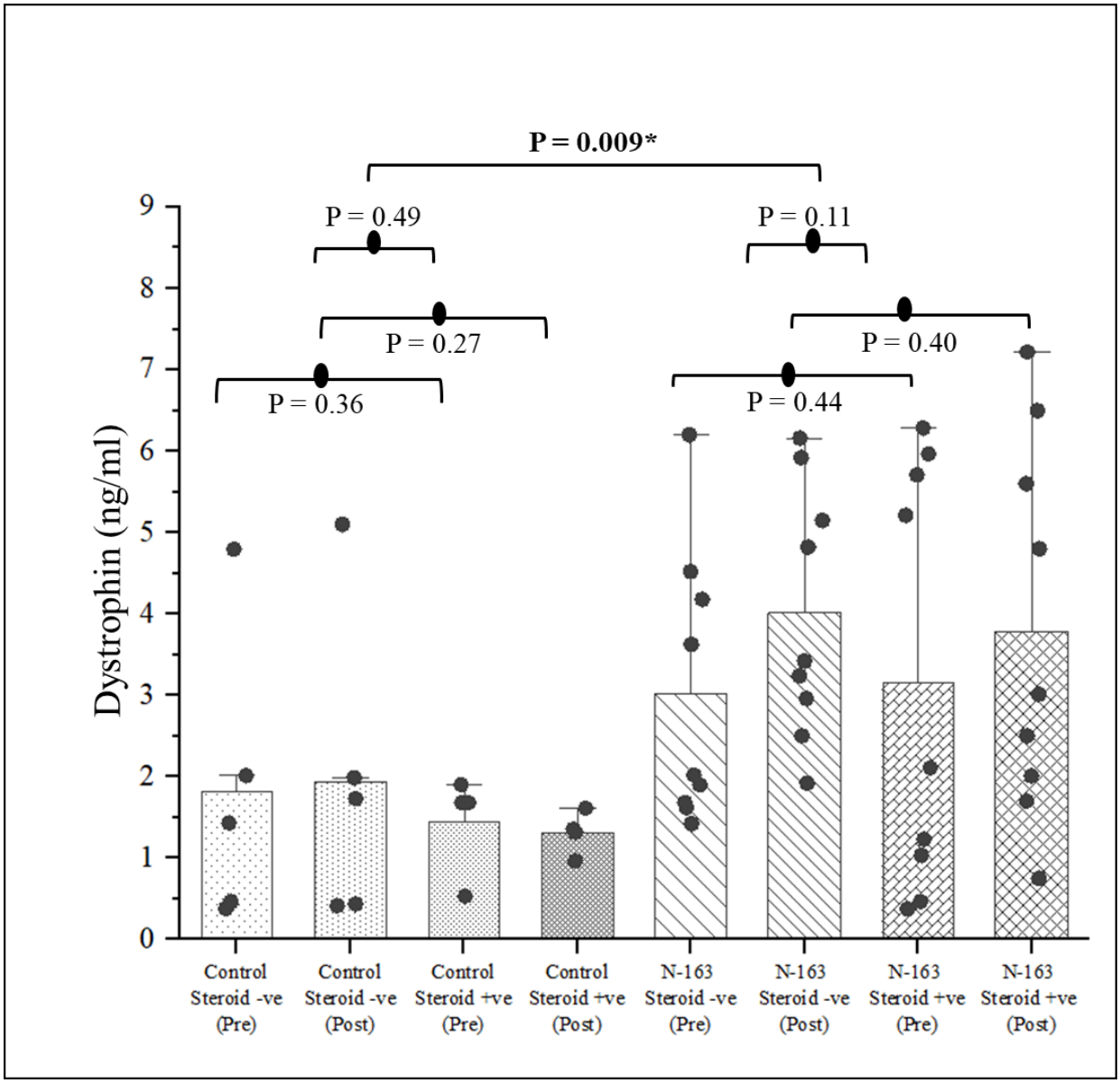
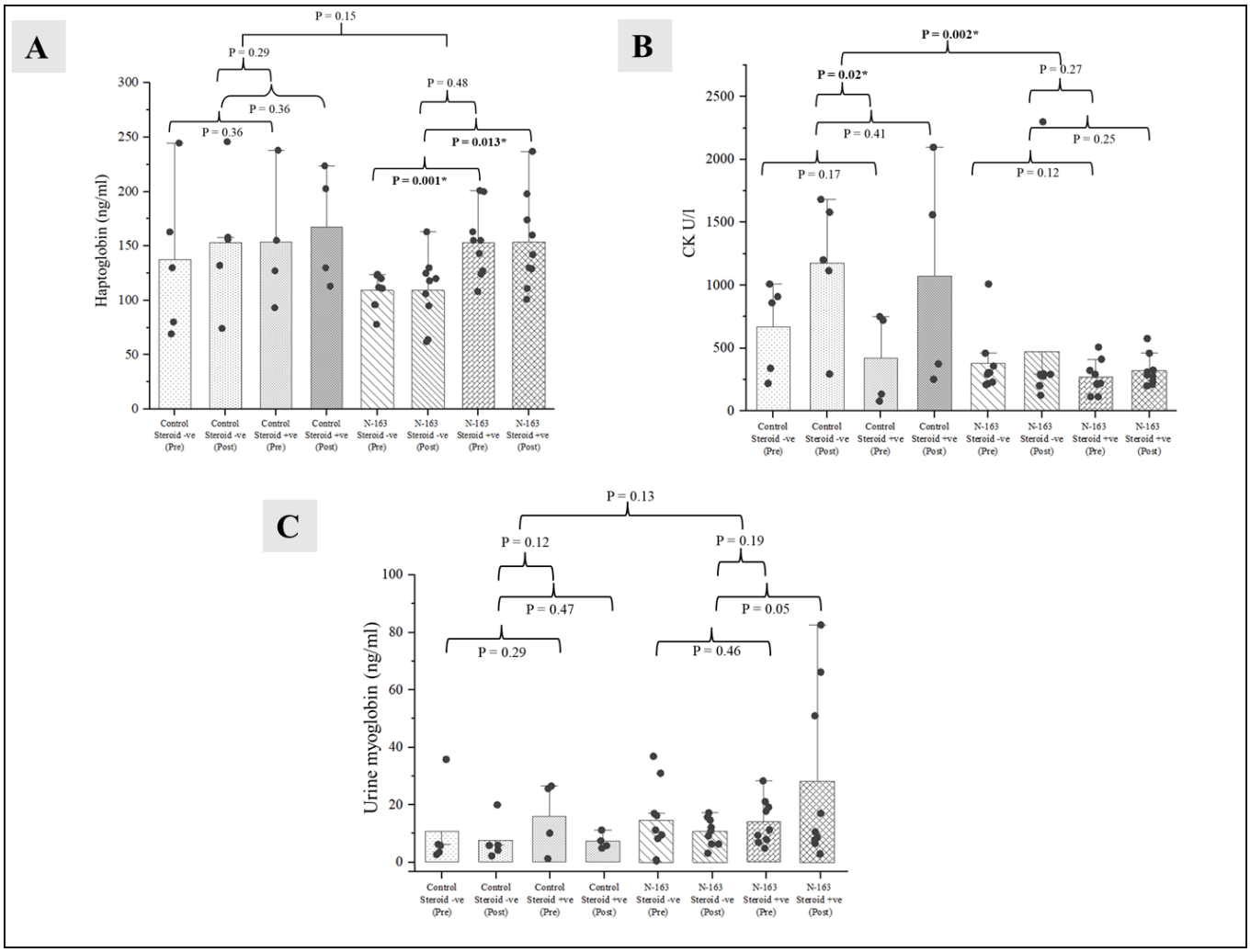


Figure 5: Levels of dystrophin showed significant increase in the N-163 Steroid -ve group compared to other groups (*p-value significance < 0.05)



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534 Figure 6: Levels of A. haptoglobin; B. CK and C. urine myoglobin in various groups of the

535 study (*p-value significance < 0.05)

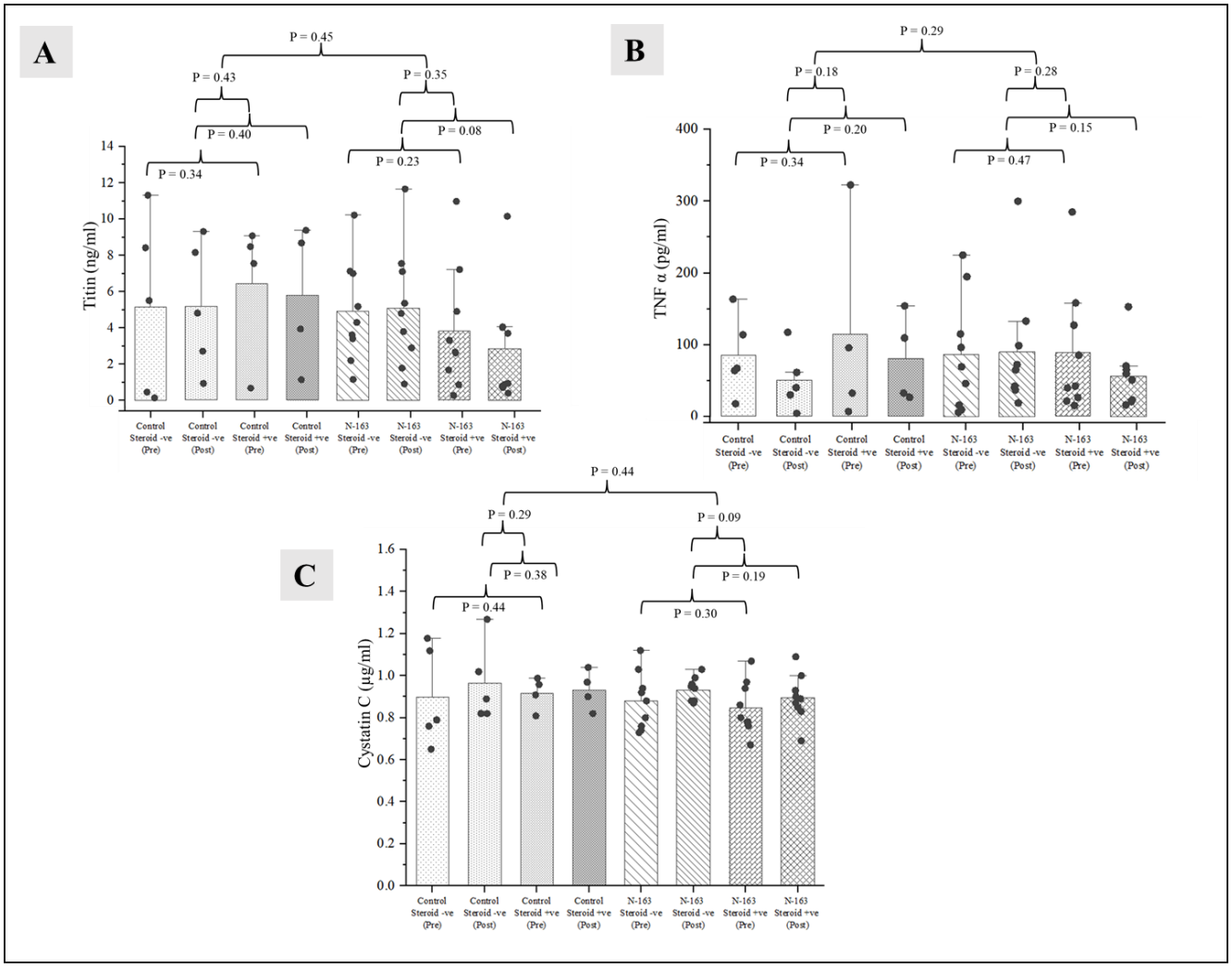

Figure 7: Levels of A. titin, B. TNF-α and C. cystatin C in various groups of the study (*p-value significance < 0.05)

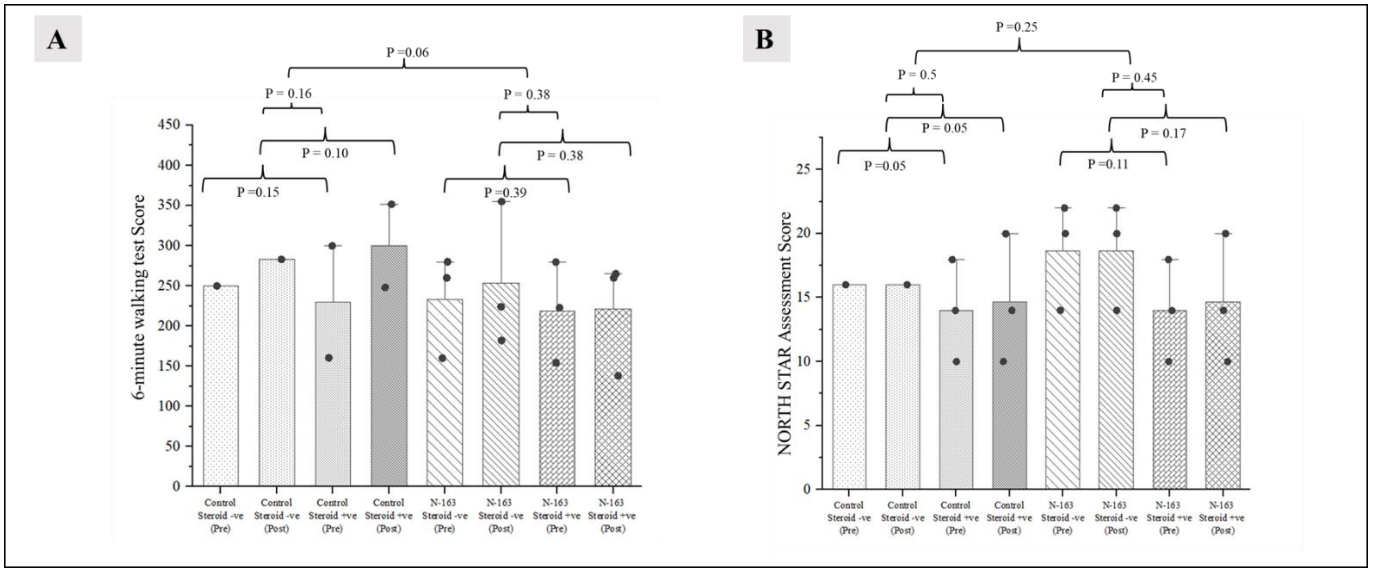

Figure 8: 6MWT and NSAA results in various groups of the study (*p-value significance < 0.05)