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Perspective

Nitric oxide donor and non steroidal anti inflammatory drugs as a therapy for muscular dystrophies: Evidence from a safety study with pilot efficacy measures in adult dystrophic patients

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ABSTRACT

This open-label, single centre pilot study was designed to evaluate safety and tolerability of the combination of the drugs isosorbide dinitrate, a nitric oxide donor, and ibuprofen, a non steroid anti-inflammatory drug, in a cohort of adult dystrophic patients (Duchenne, Becker and Limb-Girdle Muscular Dystrophy). Seventy-one patients were recruited: 35, treated with the drug combination for 12 months, and 36 untreated. Safety and adverse events were assessed by reported signs and symptoms, physical examinations, blood tests, cardiac and respiratory function tests. Exploratory outcomes measure, such as the motor function measure scale, were also applied.

Good safety and tolerability profiles of the long-term co-administration of the drugs were demonstrated. Few and transient side effects (i.e. headache and low blood pressure) were reported. Additionally, exploratory outcomes measures were feasible in all the disease population studied and evidenced a trend towards amelioration that reached statistical significance in one dimension of the MFM scale. Systemic administration of ibuprofen and isosorbide dinitrate provides an adequate safety margin for clinical studies aimed at assessing efficacy.

1. Introduction

Muscular dystrophies have a complex pathogenesis since the original genetic defect leads to a host of concurrent pathogenic events. Despite substantial progress in understanding the pathophysiological bases of these diseases, no pharmacological therapies have been identified that increase muscle strength, other than corticosteroids. Several studies provide reliable data on the benefit of both prednisone/prednisolone and deflazacort [1–4].

The potential beneficial effects of corticosteroids include inhibition of muscle proteolysis, stimulation of myoblast proliferation, increase in myogenic repair, anti-inflammatory immunosuppressive effects, reduction of cytosolic calcium concentrations [5] and up regulation of utrophin [6].

Several side effects, however, limit steroids usefulness [1]. New therapies may not be able to substitute entirely the steroids but may complement them and thus limit their use and/or reduce their dosages. For muscular dystrophies in adulthood, there have been only few small clinical trials and none involving novel therapeutic drugs or drug combinations [7–13]. We recently carried out studies in the mdx and α-sarcoglycan-null mouse models of dystrophy combining nitric oxide (NO) release and non steroidal anti-inflammatory (NSAID) activity, using the NO-releasing NSAID compound HCT1026 (nitroflurbiprofen), a combination of the NO donor isosorbide dinitrate (ISDN) and the NSAID ibuprofen or a dual compound releasing NO and ibuprofen for up to 12 months [14–16]. In all studies the results show that a combination of NO and NSAID activities slows disease progression by reducing inflammation, enhancing activity of endogenous stem cells and preventing muscle wasting. The beneficial effects were persistent, while in animals treated with ISDN or ibuprofen alone

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beneficial effects were significantly less and transient. No toxic effects were registered. In addition, in terms of therapeutic outcome, combining NO release and NSAID activity was significantly more effective than the corticosteroid prednisolone. We also found that this pharmacological approach enhances fourfold homing and engrafting of arterially-delivered donor mesoangioblast stem cells, which is of importance in perspectives of combining stem cell and pharmacological approaches to yield synergic therapeutic effects [14].

Several mechanisms of action may synergise to the observed beneficial action of the combination of NO and NSAIDs. One is limiting local inflammation, which is now recognised to play a significant role in fibres destruction and in progression of muscular dystrophies [17,18]. Indeed, DNA microarray or biochemical data show that inflammatory mediators/effectors dominate the expression profile of muscles from the mdx mouse [19].

A second mechanism of action involves the beneficial effects of NO on skeletal muscle. NO stimulates muscle regeneration acting on survival, activation and differentiation of satellite cells, the mononuclear progenitors of myocytes, able to form new fibres [20–22]. Furthermore, NO enhances bioavailability of nutrients to muscle, as well as energy generation through both glycolysis and mitochondrial biogenesis [23–27]. Finally, NO enhances the ability of myogenic stem cells to engraft to the dystrophic muscle, their resistance to the damaging environment of the dystrophic muscle and their ability to differentiate into myogenic cells [22,28,29]. Given these preclinical evidence results, we decided to explore if the combination of NO release and NSAID activity is safe and useful in human dystrophy.

This article describes a clinical trial primarily aimed at assessing the tolerability and safety of the combination of NO donor ISDN and the NSAID ibuprofen. The biological activity of the drug combination through exploratory outcome measures, such as the motor function measure scale (MFM), was also explored.

2. Materials and methods

We performed an open-label single-centre clinical trial with an historical control group, with a 12 months follow up.

The study was approved by the local Ethics Committee and all patients (and parents) gave written informed consent before participation in the study. The patients were informed of the preliminary published results (preclinical data), the objectives, the study design, risks and benefits of participation. The study was conducted in agreement with the Declaration of Helsinki guidelines.

2.1. Dose selection and treatment

To choose the appropriate dose for the study we relied on both preclinical/clinical evidence and the known pharmacokinetic profile of the two drugs. According to the National Italian Drug Agency (AIFA) and to international drug regulatory agencies (European Medicines Agency. EMA, Food and Drug Administration, FDA) patients should be treated with 200–300 mg of ibuprofen 2–3 times per day. As more conservative approach, we decided to apply the most stringent recommendation: 200 mg of ibuprofen BID. This dose was chosen because it is the one approved for OTC in Europe [30] and far below the doses (800–1200 mg/day) approved in many European Countries for non-prescription in adults [31].

The same conservative approach was applied for ISDN. According to drug regulatory agencies (AIFA, EMA, FDA) adults patients should be treated with 20–120 mg of ISDN daily, eventually divided in different subfractions [32,33]. Accordingly, our patients were given ISDN at 20 mg/day during the first month, eventually up-titrated to 40 mg thereafter.

2.2. Patients

Patients were recruited from the ones referring to the E. Medea Scientific Institute for periodic clinical assessments.

All patients fulfilling the inclusion/exclusion criteria (see below) were recruited from April 2007 to April 2008.

Inclusion criteria were a minimum age of 16 years; certainty of diagnosis (clinical, histological and immunohistochemical, biochemical and molecular diagnosis of DMD, BMD, or one of the following forms of LGMD: 2A, 2B, 2C, 2D, 2E, or 2I) [2,3,34,35]; adequate comprehension of the purpose of the study; signing of the informed consent; presence of at least two "baseline" clinical evaluations.

Main exclusion criteria were: Ejection fraction <40% at the Echocardiogram; forced vital capacity <40% of predicted; concomitant pathologies: gastrointestinal disorders/diseases, hepatic and renal dysfunctions, psychiatric symptoms, allergies, migraine; inability or unwillingness of the patient to give written informed consent; inability to comply with evaluation procedures as assessed by investigator; inability to take capsules.

2.3. Study design

At screening evaluation, the patients were seen by one experienced neurologist (MG D'A, S G, S B). All relevant demographic, clinical and laboratory data were reported in a dedicated case record form. Patients fulfilling the inclusion criteria started treatment with ibuprofen (200 mg BID) and ISDN (20 mg/per day). Four weeks after starting treatment, ISDN was up-titrated to 40 mg/per day. Gastric protection was guaranteed in the treated arm by pantoprazole 20 mg/per day. Patients were maintained on ibuprofen/ISDN for 12 months. For each patient treated with ibuprofen and ISDN (case), one patient treated conservatively (control) who satisfied the same inclusion/exclusion criteria was identified among the same population of patients with muscular dystrophy. Reference cases and controls were matched with cases for gender, age and specific muscular dystrophy (DMD, BMD, LGMD).

Cases and controls were monitored periodically up to the study end (12 months after the screening visit) as specified below.

2.4. Assessments

All patients had at least one preliminary assessment, 6 months before baseline, as well as a baseline assessment immediately before beginning treatment (T0). Subsequently, they were evaluated 1 (T1), 3 (T3), 6 (T6) and 12 months (T12) after the start of the treatment.

The protocol evaluation which was applied consisted of full physical examination (including the measurements of vital signs), neurological examination, manual muscle testing (a total of 18 muscle groups were examined on both sides, testing limb movement around the neck, shoulders, elbows, wrists, hips, knees, and ankles) with the application of the Medical Research Council score (MRC), application of the motor function measure (MFM scale) [36–38], evaluation of cardiac function via Echocardiogram, 24 h electrocardiogram (ECG) registration and blood pressure measurements, and evaluation of the respiratory function via spirometry and oxyhaemoglobin saturation measurements. Plasma and urine were obtained to determine renal and liver function, electrolytes levels, complete cell counts, activated partial-thromboplastin time, creatine phosphokinase (CPK). In addition, serum pro-inflammatory cytokines (Transforming Growth Factor β, TGF-β, and Interleukin 6, IL-6) were measured in patients on combined ibuprofen plus ISDN at T0, T3, T6 and T12 by ELISA kits.

Regular blood pressure measurements were recorded by the patients and/or their care-givers.

SF36 quality of life questionnaire was administered at start and after 12 months of treatment to all the patients.

2.5. Safety

Safety and tolerability were assessed through signs and symptoms reported by the patients and relatives/caregivers as well as physical examinations, vital signs measurements, blood tests, cardiac and pulmonary evaluation. The latest were performed by the same examiners, and included measurements of forced vital capacity, forced expiratory volume in 1 second through the spirometry, ECG and 24 h ECG registration.

2.6. Biological activity

Biological activity was assessed through the MFM scale, and cardiac and respiratory function testing. The MFM scale assesses the motor function of patients. The MFM consists of 32 items (tasks) classified into the following three dimensions: D1, standing and transfers; D2, axial and proximal motor capacity; and D3, distal motor capacity [37–39]. Cardiac and respiratory function evaluations were recorded also as efficacy, as well as safety measures.

2.7. Subject-reported outcomes

Health-related quality of life (HRQoL) was measured using Version 1.0 of the Short Form SF-36, which has established validity as a measure of function and well-being [39,40]. It measures HRQoL in eight domains: physical functioning, role physical, bodily pain, general health perceptions, vitality, social functioning, role-emotional and mental health. Domain-scores are calculated on a 0–100 scale with a higher score indicating better HRQoL. Scoring of the SF-36 Physical Health and Mental Health components used a norm-based approach [41].

2.8. Statistical analysis

An intention-to-treat analysis was performed using the last-observation carried forward methodology in case of missing data. Data from all enrolled patients, including dropout when available, were included in the statistical analysis. Non parametric statistical tests were used, since sample size in the two arms was relatively small, and normal distribution was not guaranteed. Primary (safety) and secondary outcome (efficacy) measures were tested by comparing the change in outcome levels between the beginning and the end of the study in treated and control groups of patients using the Wilcoxon signed-rank test in case of quantitative data, and by comparing the distribution of variables at the beginning and the end of study using the Fisher's exact test in case of categorical outcome measures. SPSS statistical package version 13.0 (Chicago, IL, USA) was used for the analysis. No formal measurement of sample size was performed because the primary outcome of the study was the safety and tolerability of the drug.

3. Results

3.1. Patients characteristics

A total of 35 patients, of whom 10 with DMD, 7 with BMD and 18 with LGMD were treated with the drug combination. A total of 36 patients, of whom 11 with DMD, 9 with BMD and 16 with LGMD not receiving the drug combination, were checked as "controls".

In the group of treated DMD boys, 6 were steroids-naïve, 5 had been treated for a mean time of 8 ± 4.3 years (deflazacort, 0.9 mg/kg/per day). In the group of "untreated" DMD boys, 6 were steroids-naïve, 5 had been treated for a mean time of 4 ± 2.1 years with steroids (deflazacort, 0.9 mg/kg/per day).

There was no significant difference between groups in any demographic-anthropometric characteristics (Table 1), and the two arms were matched as regards to the frequency of muscular dystrophy subtypes (chi-square analysis, $p = 0.82$). The two arms were also matched in terms of age, weight, height and disease duration, while a difference was found only in LGMD group in terms of age at enrolment and age of onset, which tends to be earlier in drug-active versus untreated patients (see Table 1). Twenty eight out of the 35 patients treated with the drug combination completed 12 months of treatment.

3.2. Safety and tolerability of the combination of ISDN and ibuprofen

Safety assessments, including vital signs, cardiac and respiratory evaluations showed no differences between the baseline (T0) and 12 months after the beginning of treatment (T12) in treated BMD patients; an increase of the diastolic blood pressure, however with values always within the normal range, was observed in LGMD patients (Table 2).

Likewise, no significant changes were observed in the haematological parameters tested, including red cells and platelets count, hepatic transaminases, electrolytes and glucose, with the exception of a mild reduction in white cells and creatinine occurring in LGMD treated patients and an increase of urea levels in DMD treated patients; levels which anyhow remained within the normal ranges (Table 2).

Non statistically significant differences were observed in the left ventricle ejection fraction, in the left ventricle shortening fraction and in forced vital capacity, forced expiratory volume in 1 s at the 12 month examination versus baseline in BMD and LGMD.

DMD patients, as expected by the natural evolution of the disease, showed a decrease in the lung volumes measured with

Table 1
Baseline characteristics of the patients enrolled in the study.

	DMD ($n = 21$)		BMD ($n = 16$)		LGMD ($n = 34$)	
	Treated ($n = 10$)	Untreated ($n = 11$)	Treated ($n = 7$)	Untreated ($n = 9$)	Treated ($n = 18$)	Untreated ($n = 16$)
Age, year (SD)	21.8 (4.6)	19.5 (2.0)	38.3 (10.9)	31.3 (11.2)	31.7 (12.2)	45.1 (10.3)**
Female	0	0	0	0	6	5
Male	10	11	7	9	12	11
Onset (SD)	4.2 (0.8)	3.7 (0.5)	14.7 (8.3)	10.9 (3.9)	12.4 (5.8)	26.1 (13.0)**
Duration (SD)	17.6 (4.5)	15.7 (1.7)	19.6 (8.7)	16.0 (5.6)	19.3 (8.9)	17.8 (7.2)
Weight (SD)	65.9 (25.0)	62.9 (13.7)	67.7 (8.2)	64.3 (8.8)	61.5 (14.9)	69.1 (18.5)
Height (SD)	166.8 (11.1)	166.7 (2.3)	152.2 (50.7)	169.3 (8.3)	168.2 (9.5)	173.3 (7.1)

DMD = Duchenne Muscular Dystrophy; BMD: Becker Muscular Dystrophy; LGMG = Limb-Girdle Muscular Dystrophy.
SD = standard deviation.
** $p < 0.005$.

Table 2
Summary of the main safety parameters stratified according to the dystrophic disease.

	DMD				BMD				LGMD			
	Treated		Untreated		Treated		Untreated		Treated		Untreated	
	T0	T12	T0	T12	T0	T12	T0	T12	T0	T12	T0	T12
WBC ($10^3/\mu L$)	7.3 (2.9)	6.9 (1.8)	7.1 (1.4)	7.0 (1.1)	6.1 (2.0)	5.9 (1.7)	6.6 (1.1)	6.9 (1.0)	5.7 (1.9)	5.1 (1.4)*	6.0 (2.0)	6.0 (1.6)
PLT ($10^3/\mu L$)	281 (86)	270 (74)*	221 (59)	249 (47)	226 (69)	221 (73)	258 (53)	251 (42)	241 (64)	231 (82)	250 (159)	262 (171)
RBC ($10^6/\mu L$)	4.8 (0.3)	4.7 (0.2)	4.7 (0.4)	4.8 (0.3)	4.7 (0.4)	4.8 (0.5)	4.7 (0.2)	4.8 (0.3)	4.6 (0.5)	4.6 (0.6)	4.7 (0.4)	4.7 (0.3)
Urea (mg/dL)	26.8 (10.5)	33.9 (9.6)*	28.7 (10.6)	29.5 (7.4)	34.4 (10.1)	40.8 (11.3)	31.9 (6.2)	29.0 (6.6)	32.7 (9.5)	33.3 (12.0)	33.0 (6.9)	31.9 (6.4)
Creat. (mg/dL)	0.21 (0.06)	0.16 (0.07)	0.39 (0.1)	0.36 (0.1)	0.46 (0.18)	0.43 (0.14)	0.32 (0.3)	0.32 (0.3)	0.31 (0.13)	0.19 (0.08)*	0.49 (0.1)	0.39 (0.2)^
AST (U/L)	41 (12)	43 (21)	43 (20)	44 (10)	55 (30)	54.4 (26.0)	53 (20)	59 (20)	53 (39)	51 (32)	49 (20)	56 (23)^
ALT (U/L)	75 (30)	69 (19)	71 (50)	74 (32)	95 (39)	94 (32)	111 (50)	102 (37)	81 (49)	71 (39)	93 (44)	84 (38)
BPS (mmHg)	117 (12)	113 (10)	114 (15)	124 (19)	118 (10)	123 (11)	112 (11)	113 (14)	116 (11)	118 (13)	121 (12)	121 (12)
BPD (mmHg)	75 (10)	73 (6)	71 (10)	73 (11)	75 (10)	77 (8)	70 (7)	71 (9)	72 (10)	79 (8)**	80 (14)	75 (8)
HR (bpm)	92 (8)	91 (10)	83 (10)	88 (12)	76 (13)	75 (8)	84 (6)	85 (15)	79 (8)	85 (11)	78 (8)	79 (16)
FVC (% pred)	39.5 (21.8)	30.2 (18.4)*	34.7(10.7)	26.9 (9.6)^^	89.3 (17.7)	91.3 (7.0)	97.9 (14.9)	106.5 (17.3)	71.4 (28.1)	77.8 (28.1)	83.3 (22.5)	73.4 (28.1)
FEV1 (% pred)	37.3 (22.5)	32.0 (19.1)	34.6 (10.0)	27.1 (10.2)^^	91.4 (20.71)	88.5 (2.1)	104.4 (16.9)	114.0 (19.0)	73.9 (25.9)	77.9 (30)	81.6 (19.2)	74.9 (27.9)
LV EF (%)	51.7 (10.3)	51.9 (9.1)	47.6 (11.8)	43.5 (12.8)	53.9 (12.8)	60.8 (5.8)	52.2 (10.6)	53.8 (10.5)	63.2 (4.5)	64.0 (5.0)	62.3(6.2)	65.0 (6.6)

Data were expressed as mean (SD).
DMD = Duchenne Muscular Dystrophy; BMD: Becker Muscular Dystrophy; LGMG = Limb-Girdle Muscular Dystrophy.
WBC = White blood cells, PLT = platelet count; RBC = Red blood cells; SBP = Systolic blood pressure; DBP = Diasystolic blood pressure; AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; FEV1 = Forced expiratory volume in 1 s; FVC = Forced vital capacity; LV EF = Left ventricle ejection fraction; HR: heart rate.
% pred: percentage predicted values; T0 = baseline; T12 = 12 months after start of the treatment.
p value represent the change between T12 and baseline of the study in treated and untreated patients.
* $p < 0.05$ in treated patients.
** $p < 0.005$ in treated patients.
^ $p < 0.05$ in untreated patients.
^^ $p < 0.005$ untreated patients.
SD = standard deviation.

Table 3
Adverse events recorded during the study.

	Total	DMD	BMD	LGMD
Headache[a]	19	6	5	8
Precordial pain (negative cardiological check)	2	0	0	2
Epigastric pain[b]	5	1	1	3
Lower limbs edema	6	1	1	4
Orthostatic hypotension	5	2	1	2
Meteorism, digestive disturbances	5	3	1	1
Dermatitis	2	0	0	2

DMD = Duchenne Muscular Dystrophy; BMD: Becker Muscular Dystrophy; LGMG = Limb-Girdle Muscular Dystrophy.

[a] 17 patients present very mild headache, at the beginning of the treatment, with spontaneous decreased in one week.

[b] Advantage from increase of pantoprazole from 20 to 40 mg/day.

the force vital capacity predicted score. Cardiac function in treated DMD patients was stable (Left Ventricle Ejection Fraction: 51.7 ± 10.3 at baseline versus 49.7 ± 9.1 at the end of the study), while in the untreated DMD patients there was a non significant decrease from T0 to T12 (47.6 ± 11.8 versus 43.5 ± 12.8).

For the most part (28 out of 35 patients) the drug combination was well tolerated and drug-related side effects mild and transient: headache in the first 7–10 days of treatment (a known side effect of ISDN), transient epigastric pain, lower limb oedema, orthostatic hypotension, dermatitis, precordial pain (with negative cardiological check) (Table 3).

Four subjects with LGMD discontinued the therapy for the following adverse drug reactions: one, after 15 days because of persistent headache; one, after 6 months, because of dermatitis; two due to lack of compliance to the treatment respectively after 1 month and 6 months, due to "perception of inefficacy of the drug" and "feeling of legs weakness". In addition, one DMD discontinued the therapy for repeated episodes of tachycardia, and two BMD for tachycardia and increased extrasystolic episodes recorded at the 24 h ECG (Fig. 1). The longitudinal observation of the patients who discontinued the treatment, did not show any clinical abnormalities in a 24 months follow up. No deaths occurred during the study.

3.3. Exploratory analyses on efficacy outcomes

Table 4 reports the change in outcome measures between beginning and end of the study in treated (DMD, BMD and LGMD) compared to untreated patients.

The D1 subscale score of the MFM scale increased during the study in treated and decreased in untreated ones, this difference being statistically significant ($p = 0.03$; Wilcoxon signed-rank test).

No significant differences were observed in the other subscales and in the total scores, even if there was a less evident worsening in treated versus untreated individuals; in total scores, however, this difference verged towards significance, ($p = 0.06$) (see Table 4).

We performed also subgroup analyses of the efficacy of the drug in the three groups of patients DMD, BMD and LGMD. No significant differences were observed when analyses were stratified by disease group (Table 4).

Cytokines dosage in the patients treated with ibuprofen plus ISDN showed a progressive and significant reduction in serum TGF-β concentrations, whereas not significant were the effects on IL-6 levels (Table 5).

On direct questioning, treated patients reported some subjective improvement in endurance and increased stamina. Evidence of perceived improvement via analysis of the measures of quality of life SF-36 questionnaire emerged in the comparison of the scores from baseline to the end of the study. The overall group of patients scored an increased values of general health and, in LGMD patients, the scoring were significantly different in the summary measures of total mental health (53.6 versus 54.6; p = 0.04) and physical function (38.9 versus 40.4; $p = 0.04$) (Fig. 2).

4. Discussion

The present study demonstrates that the combined administration of ibuprofen and ISDN has a good profile of safety and tolerability for a long-term treatment as the one presumably needed in chronic genetic diseases such as human muscular dystrophies.

Ibuprofen is currently indicated for relief of mild to moderate pain, for the treatment of dysmenorrhea, for relief of the signs and

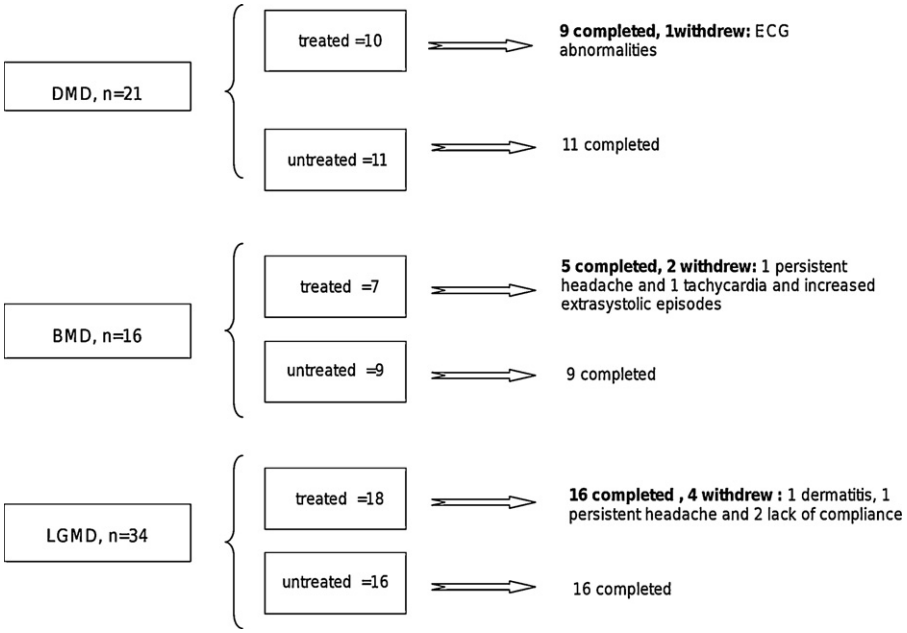

Fig. 1. Trial profile shows the breakdown of enrolled subjects, including the total number per group and the number completing the trial. DMD = Duchenne Muscular Dystrophy; BMD: Becker Muscular Dystrophy; LGMG = Limb-Girdle Muscular Dystrophy; ECG = electrocardiogram.

Table 4
Summary of the main efficacy parameters.

	T0		T12	
	Treated	Untreated	Treated	Untreated
All patients				
MFM total	47.2 (20.8)	53.4 (25.7)	47.7 (20.9)	46.0 (23.5)^
D1%	14.7 (20.6)	26.4 (31.6)	16.5 (21.1)	18.7 (26.8)*
D2%	63.3 (28.7)	64.0 (28.8)	61.9 (27.9)	56.64 (28.6)
D3%	80.4 (17.4)	82.5 (17.1)	80.9 (18.2)	78.6 (17.8)
CPK	1205.3 (1380.5)	1302.8 (1191.7)	1173.0 (1326.0)	1192.9(1265.3)
DMD				
MFM total	26.87 (12.5)	26.6 (6.2)	25.5 (12.4)	25.4 (5.9)
D1%	.51 (1.1)	0 (0)	.85 (1.8)	0 (0)
D2%	35.00 (23.8)	31.3 (8.1)	30.9 (20.9)	29.46 (7.6)
D3%	62.0 (16.7)	66.9(16.6)	61.9 (20.6)	65.9 (17.1)
CPK	1028.1 (492.7)	916.7 (541.1)	1148.75 (745.7)	803.9 (395.6)
BMD				
MFM total	62.1 (23.9)	93.4 (11.4)	69.3 (17.9)	90.0(14.1)
D1%	31.6 (29.7)	83.8 (28.1)	40.4 (28.8)	75.5 (34.6)
D2%	81.0 (27.2)	100(0)	87.1 (15.8)	100(0)
D3%	91.4 (9.2)	100(0)	91.4 (3.8)	100(0)
CPK	1314.9 (938.1)	1786.7 (1712.3)	711.3 (366.0)	1109.6 (899.6)
LGMD				
MFM total	53.6 (14.8)	64.3 (11.7)	53.8 (13.0)	58.2 (8.8)
D1%	17.3 (18.0)	32.3 (19.6)	17.8 (17.0)	25.6 (16.0)
D2%	73.1 (20.0)	80.9 (10.12)	72.21 (16.9)	76.4 (10.2)
D3%	87.6 (10.6)	90.5 (6.6)	88.9 (8.8)	87.5 (7.6)
CPK	1265.5 (1853.5)	1248.0 (1082.3)	1294.5 (1718.8)	1662.6 (1865.4)

DMD = Duchenne Muscular Dystrophy; BMD: Becker Muscular Dystrophy; LGMG = Limb-Girdle Muscular Dystrophy.
T0 = baseline; T12 = 12 months after start of the treatment. CPK: creatine phosphokinase. MFM scale: motor function measure scale; D1 subscale score = standing-position change; D2 subscale score = axial and proximal motor capacity; D3 subscale score = distal motor capacity.
p value correspond to the comparison of the change between the beginning and the end of the study in treated and untreated patients using the Wilcoxon signed-rank test. (SD) = standard deviation.
^ $p = 0.06$.
* $p = 0.03$.

symptoms of rheumatoid arthritis and osteoarthritis, for analgesia after surgical interventions and as antipyretic. In terms of adverse drug reactions ibuprofen appears to have the lowest incidence of gastrointestinal adverse drug reactions of all the non-selective NSAIDs [42]. ISDN is indicated for the prevention of angina pectoris and for the treatment of left ventricular insufficiency. Both drugs have per se an established profile of safety and are widely used as single treatment in adult population and, as far as ibuprofen, also in children. Despite the extensive use of ibuprofen and ISDN as single drug, no study was reported in which the combined administration of the two drugs was tested. Accordingly, in the present study we chose doses (400 and 40 mg per day for ibuprofen and ISDN, respectively), which were well within (and mostly below) the doses usually given to paediatric patients in the clinical practice as single treatments and associated with optimal safety profiles. In addition, the potential risk of gastrointestinal damage related to chronic ibuprofen treatment was minimised by the co-administration of a proton pump inhibitor.

Table 5
Levels of serum IL-6 and TGF-β measured in serum of patients at baseline (T0) and after 3, 6 and 12 months (T3, T6, T12) of treatment.

Time	IL-6	TGF-beta
T0	11.11 (12.43)	9.32 (1.8)
T3	5.32 (2.58)*	6.701 (1.79)
T6	8.78 (7.58)*	3.34 (1.18)*
T12	12.00 (7.02)	0.55 (0.56)**

p values correspond to the comparison of the values at indicated time point versus those of time 0 (T0) using the Wilcoxon signed-rank test.
TGF-β = transforming growth factor β; IL-6 = interleukin 6; SD = standard deviation. Cytokine values in 45 matched healthy individuals were 1.46 ± 0.79 and 4.6 ± 0.51 for TGF-β and IL-6 respectively.

The results we obtained demonstrate appropriate conditions to guarantee safety and tolerability of the treatment in a clinical long-term trial. The compliance was good and the treatment was generally well tolerated, with no significant changes in haematochemical parameters. Noteworthy but expected were reports by some patients of low blood pressure and moderate headache (at the beginning of the treatment) and transient gastric pain. These are the side effects known to occur in subjects using ISDN or ibuprofen, respectively. Nevertheless, it must be stressed that these symptoms were transient and fully recovered in almost all patients,

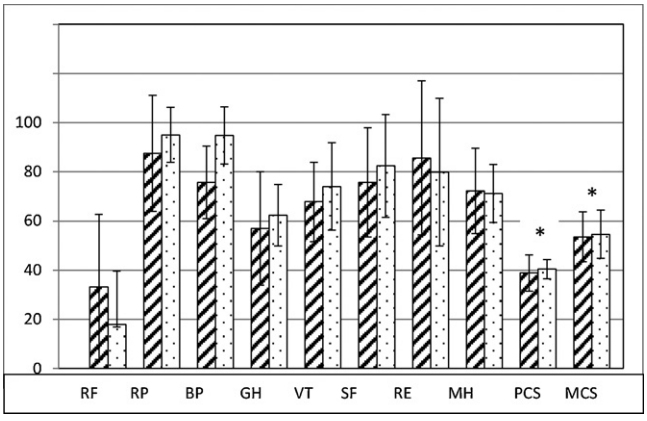

Fig. 2. SF 36 mean scores in 15 LGMD patients at baseline (T0) and after 12 months of treatment (3 patients out of 18 did not complete the questionnaire). PF: physical functioning; RP: role limitation due to physical problems; BP: bodily pain; GH: general health perception; VT: vitality; SF: social functioning; RE: role limitations due to emotional problems; MH: mental health; PCS: physical function summary measure; MCS: mental health summary measure. Column with stripes: T0; column with dots: T12; *$p < 0.05$.

not requiring treatment withdrawal. Only seven patients stopped the active treatment due to adverse events (dermatitis, persistent headache and tachycardia). Also in these cases, symptoms fully recovered after treatment withdrawal and a longitudinal observation in 24 months follow up did not show any clinical abnormality. Of importance, no target-related side effects were identified; that is, no side effects to skeletal, smooth, or cardiac muscle were found.

In the studies performed in mouse models with muscular dystrophies, the combination of NO donors and ISDN drugs had therapeutic efficacy with amelioration of strength and motor function tests together with reduction of inflammation, enhancement of activity and proliferation of endogenous stem cells and prevention of muscle wasting [14–16,22,43].

The exploratory outcome measures applied in the described pilot study (such as the MFM scale) were found to be feasible in all disease populations studied.

Although an improvement in strength and function was not demonstrated because the study was not powered for such endpoints, the efficacy of the treatment should still be underlined. The MFM D1 dimension, focused on standing and transfers items, calculated in the overall treated population, showed a significant improvement from T0 to T12. Different considerations should be done in the group of adult DMD boys, in which cardiac function in the treated group was stable, while decreasing from T0 to T12 in the untreated group, as expected by the natural history of the disease. Beside the care due to the small number of patients in each group, this observation suggests a benefit of the drug combination, in particular of ISDN, also on heart function of DMD patients via a NO-induced activation of cellular signalling [44]. These findings support the possibility of developing the combination of ISDN and ibuprofen as a therapy to reduce cardiac function deficits that may occur in adult DMD patients.

Finally, our data suggest regulation of inflammation as an important therapeutic target in muscular dystrophy [45]. We found a significant and progressive reduction in serum TGF-β concentration in patients on combined ibuprofen-ISDN treatment (nearly a 15-fold reduction at month 12 compared with basal observations).

Considering the duration of the treatment in the experimental trial, it is not unexpected that strength is stable in adults with slow course muscular dystrophy, such as BMD and LGMD; the ability to detect arrest of disease progression or minimal improvements in strength would require larger sample sizes in a study designed to look at efficacy. Altogether the findings of this study encourages further testing of NO donors and NSAIDs as a therapy in muscular dystrophy. The doses of ISDN and ibuprofen that were used here were at the lower limits for therapeutic efficacy. The possibility that higher doses of ISDN and/or ibuprofen improve the clinical outcome of dystrophic patients, still maintaining optimal tolerability, is currently being assessed.

The advantages of the treatment we propose here is that it is based on drugs that are safe, economically affordable, suitable for most dystrophic patients independently from the genetic mutation. Moreover, the capability to slow the muscular degenerations (as demonstrated in mouse models) makes these molecules useful as in both stem cell and gene therapies, the efficacy of which appears limited by the state of preservation of the muscular tissue [46–48]. The small cohort of patients (for each group of dystrophies: DMD-LGMD and BMD), the heterogeneity of the studied population, and the lack of randomisation represent the limitations of this study; however, the size of the effects found in this pilot study can be used for power analysis of future studies aiming at investigating efficacy.

Conflict of interest

All authors declare no conflict of interest related to the present work.

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