Effect of Sildenafil on Skeletal and Cardiac Muscle in Becker Muscular Dystrophy

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Objective: Patients with Becker muscular dystrophy (BMD) and Duchenne muscular dystrophy lack neuronal nitric oxide synthase (nNOS). nNOS mediates physiological sympatholysis, thus ensuring adequate blood supply to working muscle. In mice lacking dystrophin, restoration of nNOS effects by a phosphodiesterase 5 (PDE5) inhibitor (sildenafil) improves skeletal and cardiac muscle performance. Sildenafil also improves blood flow in patients with BMD. We therefore hypothesized that sildenafil would improve blood flow, maximal work capacity, and heart function in patients with BMD.

Methods: A randomized, double-blind, placebo-controlled crossover design with two 4-week periods of treatment, separated by 2-week washout was used. We assessed brachial artery blood flow during maximal handgrip exercise, 6-minute walk test, maximal oxidative capacity, and life quality; cardiac function was evaluated by magnetic resonance imaging (MRI) at rest and during maximal handgrip exercise. Muscle nNOS and PDE5 were tested with Western blotting in 5 patients.

Results: Sixteen patients completed all skeletal muscle evaluations, and 13 completed the cardiac MRI investigations. Sildenafil had no effect on any of the outcome parameters. No serious adverse effects were recorded. PDE5 and nNOS were deficient in 5 of 5 biopsies.

Interpretation: Despite positive evidence from animal models of dystrophinopathy and physiological findings in patients with BMD, this double-blind, placebo-controlled clinical study showed no effect of sildenafil on blood flow, maximal work capacity, and heart function in adults with BMD. This discrepancy may be explained by a significant downregulation of PDE5 in muscle.

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Becker muscular dystrophy (BMD) is the third most common muscular dystrophy. It has a variable phenotype with onset of symptoms from childhood to the 4th decade.[1–4] BMD is caused by mutations in the dystrophin gene leading to a truncated but partly functional dystrophin protein. Dystrophin is part of a glycoprotein complex, which connects the intracellular sarcomere to the extracellular matrix. The exact mechanism by which lack of dystrophin causes disease is not understood, but a main hypothesis is that a defect in dystrophin destabilizes the muscle cell mechanically, leading to disruption of the sarcolemma and, in turn, muscle cell death. The dystrophin-associated glycoprotein complex anchors the enzyme, neuronal nitric oxide synthase (nNOS), which is deficient in humans with dystrophinopathy[5,6] as well as in the mouse dystrophinopathy model (mdx).[5,6]

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Additional Supporting Information can be found in the online version of this article.

Blood flow in healthy muscle is controlled by sympathetic tone, but during exercise, blood supply is ensured by functional sympatholysis induced by nNOS. nNOS produces vasodilation by catalyzing the production of nitric oxide, which leads to production of cyclic guanosine monophosphate (cGMP) that mediates muscle vasodilation. Evidence indicates that loss of nNOS results in insufficient vasodilation and hence ischemia in exercising muscle of persons affected by dystrophinopathies.[7–9] cGMP is degraded by phosphodiesterase type 5 (PDE5). nNOS-induced vasodilation is deficient in mdx mice[7,10–13] and in humans with dystrophinopathy,[9,14] but can be rescued using a PDE5 inhibitor, which blocks the breakdown of cGMP, the nNOS effector. Furthermore, in mdx mice, skeletal muscle and cardiac dysfunction is reversed by PDE5 inhibitors.[15–17] In addition, a recent study of BMD patients demonstrated that sympathoadrenergic vasoconstriction in contracting muscle is increased compared to controls, and that this can be reversed by an PDE5 inhibitor.[14]

Converging lines of evidence therefore suggest that nNOS is important for blood supply to the exercising muscle, and that substitution of nNOS mediators by treatment with a PDE5 inhibitor reverses these effects. However, this has not yet been examined clinically over time in patients with dystrophinopathy.

We therefore studied the effect of a PDE5 inhibitor, sildenafil, on muscle blood flow and function in patients with BMD.

Patients and Methods

The study was approved by the local ethics committee (EudraCT number 2010–024659-10) and the Danish National Board of Health. All participants signed informed consent forms. The study was registered at ClinicalTrials.gov as NCT01350154 and monitored by the local Good Clinical Practice unit according to guidelines.

Participants

All Danish ambulatory patients with confirmed BMD $\geq$ 18 years old were invited to participate. Inclusion and exclusion criteria are listed in Table 1. Patients with all types of dystrophin gene mutations were included, as nNOS may be deficient irrespective of whether the mutation involves the nNOS binding site.[18]

Design

The study was a single-center, 12-week randomized, double-blind, placebo-controlled crossover study consisting of two 4-week treatment periods separated/followed by 2 weeks of washout/follow-up. At visit 1, participants were included (see Table 1 for inclusion/exclusion criteria) and randomized, and disease severity and medical history was recorded (Supplementary Table). Outcomes were assessed before and after each medication period; however, due to limited availability of magnetic resonance imaging (MRI), only 1 baseline heart examination was performed.

Trial Setting

The study took place at the Neuromuscular Clinic, Rigshospitalet and Department of Diagnostics, Glostrup Hospital, Copenhagen, Denmark between December 2011 and January 2013.

TABLE 1. Inclusion and Exclusion Criteria

Inclusion criteria
Age > 18 years
Diagnosis of Becker muscular dystrophy confirmed by genetic testing and/or Western blot
Reduced cardiac ejection fraction (<50%) and/or skeletal muscle weakness (MRC < 5)
If medicated with ACE inhibitor, β- or α-adrenergic receptor blocker or glucocorticoid stable dosing for >3 months
Signed informed consent
Exclusion criteria
Participation in other scientific studies within 30 days
Use of medication with nitrate or potent CYP3A4 inhibitors
Use of α-adrenergic receptor blocking agents when hemodynamically unstable
Hypotension (BP < 90/50)
Recent stroke or AMI (<6 months)
Severely affected liver function (ASAT > 500U/l), when corrected for CK elevation
Nonarthritis anterior ischemic optic neuropathy

ACE = angiotensin-converting enzyme; AMI = acute myocardial infarction; ASAT = aspartate aminotransferase; BP = blood pressure; CK = creatine kinase; MRC = Medical Research Council.

Medication

Active medication was the PDE5 inhibitor, sildenafil (20mg tablets 3× daily). This dose was chosen as it had significant vascular effects in a large clinical trial for pulmonary hypertension.[19] Tablets were taken 1 hour pre-evaluation, and subjects abstained from nicotine and caffeine intake for 12 hours before evaluation.

Outcome Measures

PART 1: SKELETAL MUSCLE FUNCTION AND BLOOD FLOW. The primary endpoint was brachial artery blood flow during maximal handgrip. Secondary endpoints were 6-minute walk test (6MWT), maximal oxidative capacity (VO_{2max}), and life quality (Short Form 36 [SF36] questionnaire).

PART 2: CARDIAC FUNCTION. The primary endpoints were resting left ventricular end-diastolic volume (LVEDV) and exercise-induced cardiac output (CO). Secondary endpoints were resting and exercise-induced ejection fraction (EF%), left ventricular end-systolic volume (LVESV), resting CO, and exercise-induced LVEDV.

Evaluations

Blood flow in the brachial artery was measured using an ultrasound Doppler General Electric (Milwaukee, WI) Logiq E9 with a 6 to 15MHz transducer in B-mode small part setting. Calculation was done using the Logiq E9 flow assessment program. Blood flow was assessed before, during, and after 2 minutes of exercise with repetitive 5 seconds of maximal handgrip and 5 seconds of rest. This exercise was chosen because BMD patients often have pronounced proximal weakness, and because this exercise paradigm is applicable to MRI.

6MWT was carried out according to guidelines.[20] VO_{2max} was assessed with an incremental cycle test. Workload was increased every 1 to 2 minutes until exhaustion.

For heart MRI we used a 1.5T MRI scanner (Siemens, Erlangen, Germany) equipped with a 32-channel cardiac-specific coil (retrospective electrocardiographic gating). Volumetric analysis of the heart chambers and through-plane flow at the aortic valve and sinotubular junction were acquired during rest and exercise. The exercise paradigm was identical to that used during brachial artery flow evaluation. Analyses were carried out using CMR42 software (Circle Cardiovascular Imaging, Calgary, Canada).

Five muscle biopsies taken previously (Subjects 7, 13, 14, 16, and 17) were assessed for nNOS (BD Biosciences, Franklin Lakes, NJ) and PDE5 (FabGennix, Frisco, TX) content by Western blot and normalized to alpha-tubulin (Developmental Studies Hybridoma Bank, Iowa City, IA). Sections from the biopsies were homogenized, run on a 10% sodium dodecyl sulfate–polyacrylamide electrophoresis gel, and blotted onto a polyvinylidene difluoride membrane. The membranes were blocked in nonfat milk, and incubated in primary and secondary antibody. Blots were visualized in a densitometer (GBox XT16; Syngene UK, Cambridge, UK), and relative content was calculated using Genetools software (Syngene UK).

T1-weighted MRI (1.5T, Siemens) of calves and thighs was obtained in 2 patients, 1 mildly and 1 moderately affected (Patients 14 and 17).

Sample Size

Before initiation of the study, no human studies using PDE5 inhibitor for dystrophinopathy had been published, but studies in mdx mice found marked effects in only 12 animals. Because these models were not applicable to our study, regular power calculation could not be done. We therefore reasoned that 12 participants would be sufficient. This assumption was supported by a study published while our trial was ongoing that found a significant PDE5 inhibitor effect in 10 BMD patients.[14] To secure 12 patients to complete the trial, we aimed at including 20 participants, but inclusion stopped at 17, because no more eligible subjects were available.

Randomization and Blinding

An independent pharmacist having no contact with the participants dispensed active and placebo tablets according to a computer-generated randomization list. Randomization was carried out in blocks of 4 with a 2:2 relationship between active or placebo treatment, and a list of sequential randomization numbers was created corresponding to inclusion number. No further restrictions or stratifications were done. Active and placebo medication were packed in identical capsules and dispensed in identical plastic containers clearly marked with "period 1" and "period 2." There was no taste, odor, or other dissimilarity between the capsules. The pharmacist was the only nonblinded person.

Statistics

A 1-way analysis of variance (ANOVA) for repeated measures was carried out for primary outcome measures. For secondary outcome measures, average values for each evaluation were calculated and then visually inspected for possible significant differences between active and placebo treatment. When significance was suspected, an ANOVA for repeated measures was performed. A significance level of $<0.05\%$ was applied, resulting in a risk of type 1 error of 0.95. As only 4 ANOVAs were carried out, no correction for multiplicity was done.

Results

Participants

Participants were recruited from December 2011 to November 2012, when no more participants were available. Forty-seven patients were screened. Ten did not fulfill inclusion criteria (1 had a pacemaker and 9 were nonambulatory), and 20 declined participation. Thus, 17 were included in the study. One withdrew consent due to anxiety after visit 2. Sixteen completed part 1 (skeletal muscle assessment). Because 2 had claustrophobia and 1 a transplanted heart, only 13 completed part 2 (cardiac assessment).

Baseline Data

Average age was 38 years (see Supplementary Table). Disease stage varied from mild affection with Medical

TABLE 2. Results of Primary and Secondary Effect Parametres

Endpoint	Baseline, Ave/SD	Placebo, Ave/SD	Δ, Placebo–Baseline	Sildenafil, Ave/SD	Δ, Sildenafil–Baseline	*p*, ANOVA	Power
Primary							
Blood flow, ml/min[a]	532/245	633/278	101	642/284	110	0.56[b]	0.67
Cardiac function							
LVEDV resting, ml	160/54	158/55	2	161/57	1	0.83	0.05
CO exercise, ml/min	7,503/1,341	7,233/1,473	30	72,263/1,012	20	0.65	0.05
Secondary							
6MWT, m	436/194	446/197		438/197		ND	
VO_{2max}, ml/kg/min	25/12	27/12		25/13		ND	
Load, Watt	94/72	88/66		93/73		ND	
Heart rate, beats/second	160/26	160/24		158/29		ND	
SF36 global score	71/28	72/28		73/26		ND	
EF% resting	58/10	60/10		58/10		ND	
EF% exercise	56/9	58/10		59/10		ND	
Resting end-systolic volume, ml	69/26	65/24		69/30		ND	
Exercise-induced end-systolic volume, ml	72/27	69/26		68/31		ND	
Exercise-induced stroke volume, ml	88/14	91/16		91/15		ND	
Exercise-induced end-diastolic volume, ml	160/31	160/33		158/38		ND	

[a]Blood flow indicates mean blood flow in the brachial artery during maximal handgrip exercise, average of the 2 baselines.
[b]Active versus placebo.
6MWT = 6-minute walk test; ANOVA = analysis of variance; Ave = average; CO = cardiac output; EF% = ejection fraction; LVEDV = left ventricular end-diastolic volume; SD = standard deviation; SF36 = Short Form 36 (life quality questionnaire; higher score means better function); VO_{2max} = maximal oxidative capacity; Watt = workload at VO_{2max}.

Research Council (MRC) 4+ in just 1 region to pronounced generalized involvement and limited walking ability. The genotype was relatively homogeneous, as 12 had deletion in exons 37 to 48. Pronounced differences in cardiac and skeletal muscle phenotypes with the same mutation were noted in the nine 30- to 62-year-old patients with deletion of exons 45 to 48 (see Supplementary Table).

Analysis

The primary analysis was intention to treat and involved all patients who were randomly assigned; however, baseline data from 1 subject who withdrew consent and had no postmedication assessments were excluded. In addition, 3 persons only participated in part 1. Hence, overall cardiac data from 13 subjects and skeletal muscle data from 16 subjects were analyzed.

In part 1, data were missing partly or completely from 8 of 256 data points. Four of the 8 missing data points were baseline evaluations, and the other baseline was therefore used for evaluation. Of the remaining 4 missing data points, 1 lacked flow data after exercise, 1 lacked data from the last of the 6 flow assessments during exercise, and a third lacked the complete flow examination after active treatment. Finally, 1 patient lacked 6MWT after placebo. These data were analyzed in the ANOVA as missing data.

Study Part 1: Skeletal Muscle Evaluation

The primary outcome measure, average flow during exercise, increased during exercise after sildenafil as well as placebo treatments compared to baseline, but there was no difference between sildenafil and placebo (Table 2). In addition, a plot of average flow curves during the

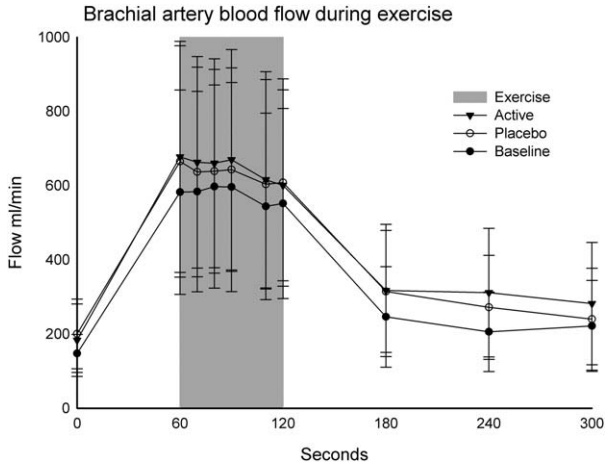

FIGURE 1: Brachial artery blood flow during exercise. Participants exercised by maximal clenching the hand for 5 seconds and relaxing the hand for 5 seconds, allowing for measurements to be made.

complete 5-minute assessment period (Fig 1) did not suggest any effect of sildenafil before or after exercise. There was a direct relation between handgrip force and increase in flow (Fig 2), but no apparent relation between handgrip force and response to sildenafil.

The secondary effect parameters, 6MWT, VO_{2max}, and the SF36, also did not differ between sildenafil and placebo treatments (Tables 2 and 3).

Study Part 2: Heart Muscle Evaluation

Regarding the primary outcome measures, no effect of sildenafil was noted on resting LVEDV and CO during exercise (see Table 2 and Fig 2).

Also, sildenafil did not have an effect on the secondary effect parameters, resting and exercise-induced EF% and LVESV, resting CO, and exercise-induced LVEDV (see Table 2).

Western Blot

Five patients (Patients 7, 13, 14, 16, and 17) had Western blotting of muscle nNOS and PDE5 performed. All were deficient in nNOS (0.50 ± 0.08; normal = 1), but PDE5 (0.18 ± 0.06) was consistently more reduced (Fig 3A, B). This deficiency was not clearly related to disease severity.

MRI

Two participants (Patients 14 and 17) had MRI performed on the lower extremities (see Fig 3C). Subject 14 had normal strength; his 6MWT was 650m and his VO_{2max} was 45ml/kg/min. Not surprising, his MRI was normal.

Subject 17, conversely, had weakness down to MRC 3− in his lower extremities and a waddling gait. His 6MWT was 450m and his VO_{2max} was 20ml/kg/min. In agreement with these results, his MRI revealed pronounced atrophy and fatty infiltration in the lower limb musculature (see Fig 3C).

Side Effects

Side effects were recorded at 2 and 4 weeks of treatment. Dyspepsia and headache were reported by 5 participants

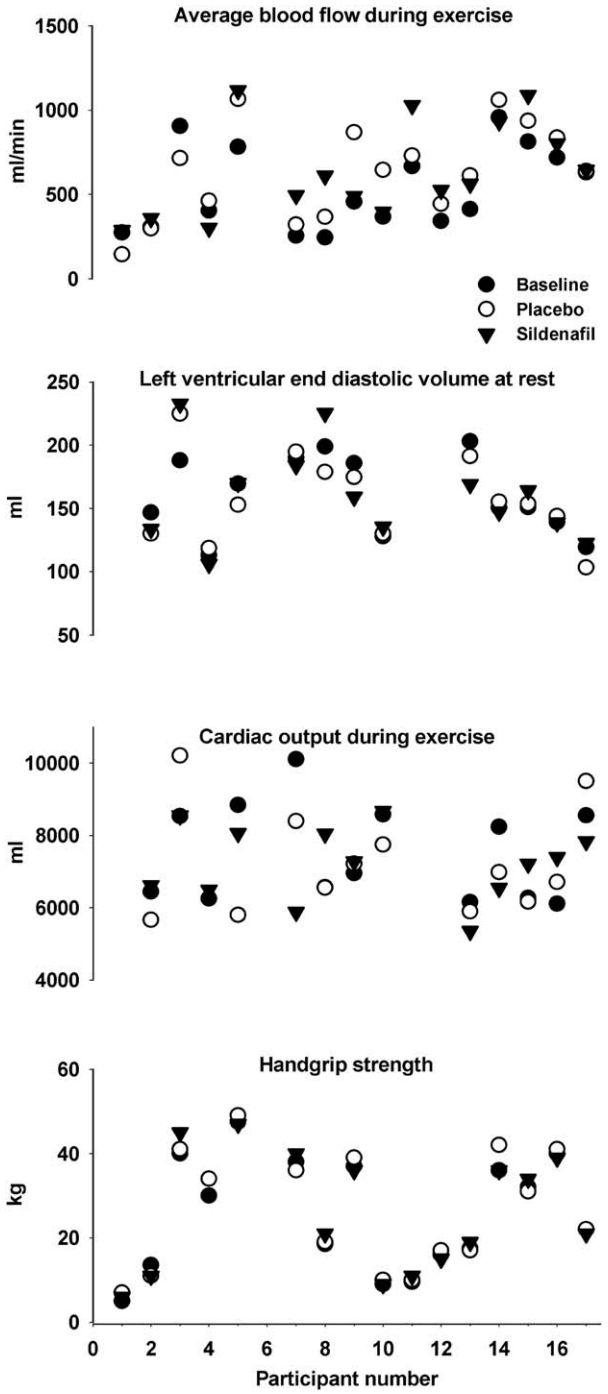

FIGURE 2: Individual primary effect parameters: arterial blood, left ventricular end-diastolic volume at rest, cardiac output, and handgrip strength in each patient with Becker muscular dystrophy at baseline and during placebo and sildenafil treatments.

TABLE 3. Results of SF36 Subcategories

SF36 Subcategories	Baseline, Ave/SD	Placebo, Ave/SD	Sildenafil, Ave/SD	*p*, ANOVA
Physical functioning	49/30	51/28	47/31	ND
Role limitations due to physical health	69/36	75/35	81/25	0.32
Role limitations due to emotional problems	85/28	77/38	90/23	ND
Energy/fatigue	61/18	63/17	63/18	ND
Emotional well-being	78/18	77/18	76/17	ND
Social functioning	86/18	90/16	86/16	ND
Pain	87/13	91/12	86/12	ND
General health	53/25	54/27	53/25	ND

ANOVA = analysis of variance; Ave= average; ND = not determined; SD = standard deviation; SF36 = Short Form 36 (life quality questionnaire; higher score means better function).

during sildenafil, and 1 reported headache during placebo treatment. To maintain blinding, effects on erectile function was not asked for; however, 4 patients volunteered improved erectile function during sildenafil and none did during placebo.

Discussion

The main finding of the present study is that the PDE5 inhibitor, sildenafil, does not improve (1) blood flow to exercising muscle, (2) resting LVEDV and exercise-induced CO, (3) the 6MWT, (4) VO_{2max}, or (5) life quality in patients with BMD. These findings are surprising, considering the positive effect of sildenafil in the mdx mouse and physiological studies in humans with dystrophinopathies, and underline how difficult it often is to transfer such promising, preliminary findings to a clinical setting. The observations may be explained by another novel finding of this study, namely that patients were deficient in PDE5, which is necessary for sildenafil to work. Our results are in line with a simultaneous study examining the effect of sildenafil on heart function in Duchenne muscular dystrophy and BMD. That study was stopped prematurely because an interim analysis suggested a decline in cardiac function on sildenafil (Leung et al, page x of this issue). The decline was only observed in a few patients, and collectively, the studies are not statistically different, demonstrating the lack of efficacy of sildenafil on heart function in dystrophinopathies.

Other explanations for the lack of effect of sildenafil may be considered. Our participants had variable disease severity from almost normal function to severe motor disability. One possible explanation for the lack of sildenafil effect could be a floor effect, whereby severely affected patients are unable to improve function on sildenafil due to irreversible structural muscle damage. The opposite could also be argued, namely that mildly affected individuals had a ceiling effect and could not improve further. To investigate these possibilities, we graphically plotted individual participants' primary outcomes relative to their handgrip strength (see Fig 2). From this plot, it is evident that there is no systematic lack of effect of sildenafil in mildly or severely affected patients, suggesting that ceiling or floor effects could not explain the lack of positive effect on a group level.

Another consideration might be whether measurement of brachial artery flow reflects muscle arteriolar flow. Brachial artery blood flow to the arm supplies bone and skin, as well as muscle. Blood flow to the bone presumably does not increase in response to exercise. Skin flow increases minimally during exercise at normothermia,[21] but as skin does not have nNOS, any increase would be leveled out by the placebo condition. The increase in brachial artery flow during exercise, therefore, almost exclusively reflects the increase in muscle blood flow. As there is no shunt system in the muscle, blood must pass through muscle arterioles.

In the present study, we did not include healthy controls, as it was a crossover study in which every subject was his own control. A previous study from our group, however, examined blood flow in healthy subjects with an identical technique, and found that the resting flow, like that in our BMD patients, was 200ml/min.[22] At a lower intensity of exercise, pressing about 12kg during each handgrip contraction, healthy subjects reached a blood flow of 600ml/min, which was equivalent to the flow reached in our BMD patients. However, our patients worked at a considerably higher workload (about 20kg per squeeze), which suggests an impairment of

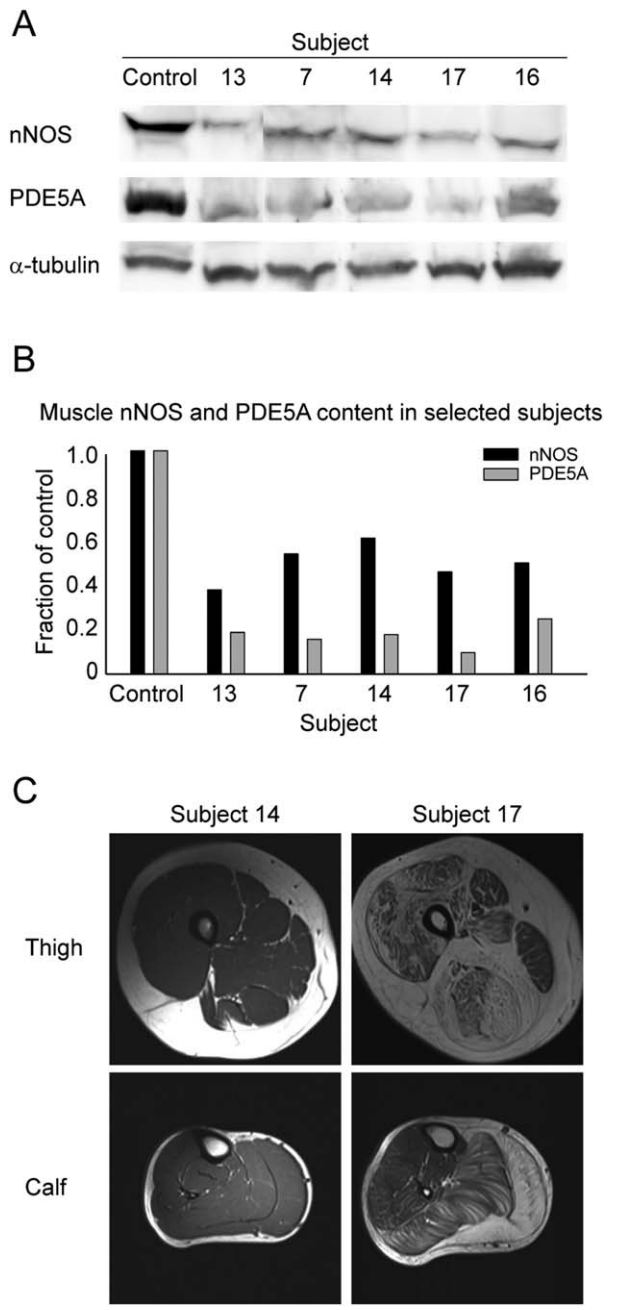

FIGURE 3: Western blotting of neuronal nitric oxide synthase (nNOS) and phosphodiesterase type 5A (PDE5A), and leg magnetic resonance imaging (MRI). (A) nNOS and PDE5A expression in muscle of 5 patients with Becker muscular dystrophy and 1 healthy control. α-Tubulin was used as a protein loading control. (B) Graphic presentation of the PDE5A and nNOS content assessed by Western blotting. (C) MRI of right thigh and calf in Subjects 14 and 17, showing fatty infiltration and atrophy especially in the vastus musculature, adductor magnus, and gastrocnemius muscle.

exercise-induced increase in blood flow. This is in agreement with the notion that exercise-induced blood flow is impaired in dystrophinopathies.

It could also be argued that with a power down to 0.05, outliers can distort interpretation in small cohorts like ours. However, as apparent from Figure 2, outliers were distributed randomly in baseline, placebo, and sildenafil evaluations, and outliers therefore likely do not contribute to a lack of effect.

We used a sildenafil dose of 20mg 3× daily for 4 weeks. A large study of the effect of sildenafil in pulmonary hypertension used the same dose and found significant effect on the vasculature.[19] In addition, our subjects reported side effects specific for sildenafil indicating a systemic effect. We therefore believe that the dose of sildenafil used was sufficient. Effect of PDE5 inhibitors in BMD has only been studied once before. In that study, a single dose of a different PDE5 inhibitor (tadalafil) was used.[14] Comparison with our study is therefore not possible.

nNOS is downregulated or lost completely in dystrophinopathy,[18,23] and in accordance with this, we found deficiency of nNOS in all studied biopsies from patients. The level of nNOS loss may depend on whether the 16/17 rod domain in the dystrophin gene is lost, as this region is essential for nNOS function.[24,25] Thirteen of our patients had mutations affecting this region (Patients 1, 2, 4, 5, 8, 9, 10, 11, 12, 13, 14, 16, and 17). However, as seen in Figure 2, the response to sildenafil was independent of mutation site, and Western blot also suggested a similar downregulation of nNOS irrespective of where the mutation was localized (see Fig 3A, B).

Figure 3 shows that the subject with normal muscle MRI expresses more nNOS, suggesting a relation between muscle nNOS content and clinical phenotype. A previous study supports this, but the relationship is not straightforward.[18]

nNOS exerts its effects via the second messenger cGMP, which is broken down by PDE5. Sildenafil, which is a PDE5 inhibitor, therefore is dependent on the presence of PDE5 to exert nNOS substitution effects. It has been shown that activity of cGMP hydrolyzing phosphodiesterase (PDE1, PDE2, PDE5) is increased in the mdx mouse,[26] but no reports of the content of phosphodiesterase in humans with dystrophinopathy exist. We therefore examined PDE5 expression on Western blot. Surprisingly, and in contrast to the findings in rodents, PDE5 was deficient in 5 of 5 BMD patients. This is of major importance, as this finding indicates that the physiological target for our treatment is impaired. It may explain the lack of effect of sildenafil in humans with BMD, and at the same time explain the effect in rodents.

A recent study demonstrated that endothelial NOS (eNOS) also contributes significantly to functional sympatholysis.[27] However, because eNOS, like nNOS, exerts its effects via cGMP, it is also affected by the severe loss of PDE5.

Why then did a previous study find an effect of PDE5 inhibition on flow regulation in humans with

BMD? Besides the difference in PDE5 inhibitor used, the physiological terms under which blood flow was studied differed fundamentally. We studied blood flow in response to regular handgrip exercise. In the study by Martin et al, blood flow was studied after a high sympathetic tone was provoked experimentally by enclosing the lower body in a negative pressure chamber, inducing a lower body negative pressure of −20 to −25mmHg.[9,14] This important variation in design between our study and theirs likely explains the discrepancy in results.

In conclusion, we found a lack of effect of the PDE5 inhibitor sildenafil on a broad range of clinical manifestations of BMD. This differs from previous findings in mdx mice. The discrepancy is most likely explained by PDE5 deficiency in humans with dystrophinopathy, which contrasts with the upregulation described in the mdx mouse model.

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Potential Conflicts of Interest

C.K.: speaking fees, Bristol-Myers Squibb. J.V.: consultancy, board membership, travel expenses, Genzyme/Sanofi.

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