UCL

EVOLVING NATURAL HISTORY IN DUCHENNE MUSCULAR DYSTROPHY: IMPLICATIONS FOR STANDARD OF CARE AND EXPERIMENTAL THERAPIES

MD (Res)

Valeria Ricotti, MRCPI, MRCPCH

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To all DMD boys and their families,

who have inspired this work.

Statement of originality

I, **Valeria Ricotti** confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Duchenne muscular dystrophy (DMD) with an average global incidence of 1:5000 is an X-linked recessive disease, caused by mutations in the DMD gene encoding dystrophin. Lack of dystrophin isoforms results in progressive muscle weakness and cardiomyopathy, leading to loss of ambulation and premature death secondary to cardiac/respiratory complications. At present, there is no curative treatment. However, implementation of standards of care has significantly shifted life expectancy and the natural history of DMD has considerably evolved. Moreover, a number of promising therapeutic approaches are under development, some reaching phase II-III clinical trials. These experimental therapies will further contribute to the transformation of the disease trajectory.

The projects of my thesis intended to address specific research questions, which have an impact not only on the clinical care of DMD patients, but also advice on clinical trial design. I studied the effect of steroid therapy on the motor function in DMD boys >7 years, more specifically profiling benefits and side effects of the most commonly used regimens: intermittent and daily prednisolone. I analysed the impact of starting steroids at an earlier age than what is standard of care. I explored the role of different dystrophin gene (*DMD*) genotypes in the motor progression of the disease, further defining the genotype-phenotype correlations. All results obtained are of particular interest for clinical trials of pharmaco-gene therapies targeting specific *DMD* mutations.

Dystrophin isoforms also play an important role for the CNS and their lack causes morbidity in DMD. My investigations expanded the genotype-phenotype profile specifically in relation to neuropsychiatric co-morbidities in DMD. In

conjunction with the CNS profile of DMD, I characterized abnormalities of retinal function and developed electroretinography as a potential and non-invasive CNS endpoint for future clinical trials.

Addressing the non-ambulant DMD population, I studied quantitative magnetic resonance imaging and novel functional measures of the upper limb. These results allow for the first time to evaluate disease progression and response to treatment in non-ambulant DMD. All the results obtained in this thesis therefore enlarge our knowledge of the disease evolution under current standard treatment and contribute to trial readiness by developing new endpoints.

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For their contribution to data collection, I wish to acknowledge both the UK NorthStar Clinical Network and the Italian Clinical Network (a full list of participants can be found in the appendix)

Finally, this thesis would have not been possible without all the Duchenne patients and their families who courageously engage in research activities.

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List of abbreviations

10mRT	10-metre-run-test
3Di-sv	Developmental, Diagnostic and Dimensional Interview – short version
6-MWD	6-minute-walk distance test
AD	Prednisolone on alternate days
ADHD	Attention Deficit and Hyperactivity Disorder
AO/AONs	Antisense oligomers/ antisense oligonucleotides
APL	Abductor pollicis longus
ASD	Autistic spectrum disorder
BMD	Becker Muscular Dystrophy
BMI	Body mass index
CBCL	Child Behavioural Check List
CI	Confidence intervals
CK	Creatine kinase
CNS	Central nervous System
DFZ	Deflazacort
DMD	Duchenne muscular dystrophy
DMD	Duchenne muscular dystrophy;
DP	Daily prednisolone,
EBD	Emotional and behavioural disorders
ECRLB Br	Extensor carpi radialis longus/brevis and the brachioradialis
ECU	Extensor carpi ulnaris
ED	Extensor digitorum
EDM	Extensor digiti minimi
EK2	Egen Klassification interview version 2
EPL	Extensor pollicis longus
ERG	Electroretinography
FCR	Flexor carpi radialis
FCU	Flexor carpi ulnaris
FDP	Flexor digitorum profundus and flexor pollicis longus
FDS	Flexor digitorum superficialis and palmaris longus
FF	Fat fraction;

FVC%	Forced Vital Capacity percentage
GAI	General Ability Index
GC	Glucocorticoids
GOSH	Great Ormond Street Hospital
ID	Intellectual difficulties/disability
IP	Intermittent prednisolone 10-dayson/10-days-off
ISCEV	International Society of Clinical Vision Society
LOA	Loss of ambulation
LVSF%	Left Ventricular Shortening Fraction percentage
MLPA	Multiplex Ligation-dependent Probe Amplification
MRI	Magnetic resonance imaging;
MRS	Magnetic resonance spectroscopy;
NSAA	NorthStar Ambulatory Assessment
NSCN	NorthStar clinical network (for paediatric neuromuscular disease)
OCD	Obsessive–compulsive disorder
OPs	Oscillatory potentials
PUL	Performance of upper limb
RCPM	Raven's Coloured Progressive Matrices
RCTs	Randomised control clinical trials
RSB	repetitive and stereotyped behaviour
RSI	reciprocal social interaction
SCDC	Social Communication Disorder Checklist
SD	Standard deviation
SW	Switchers
VCI	Verbal Comprehension Index
WISC-IV	Intelligence Scales for Children- Fourth Edition
WMI	Working Memory Index
WPPSI-III	Wechsler Preschool and Primary Scale of Intelligence – third edition

CHAPTER 1: INTRODUCTION TO DUCHENNE MUSCULAR DYSTROPHY

Duchenne muscular Dystrophy (DMD, OMIM 310200) is the most common fatal childhood-onset condition, with a global prevalence of 1.7-4.2 per 100,000.[1, 2] DMD is an X-linked recessive condition, caused by mutations in the dystrophin encoding *DMD* gene. Lack of dystrophin products results in progressive muscle weakness, leading to loss of ambulation and premature death secondary to cardiac and respiratory complications. At present, there is no curative treatment.

Revised incidence

The commonly reported average incidence of DMD is 1:3500 live males.[3] However, these figures have recently been revised, supporting that the average global DMD incidence is now closer to 1:5000 live males,[4-7] with the drop being attributed to both reproductive planning within DMD families and medical advances since the 1990s.[5]

Clinical features

Affected children are diagnosed between 4 and 5 years of age,[8-10] with some children being diagnosed as late as 9 years of age, figure which has changed little over the last 3 decades. The classic clinical presentation includes the following: delayed gross motor milestones, frequent falls, abnormal gait, calf hypertrophy, frequent falls and muscle cramps. These features may present in isolation or in combination with language delay, intellectual disability, and/or behavioural problems. The classic manoeuvre, which reveals the underlying proximal weakness is known as "Gowers manoeuvre" and describes the compensatory mechanism of climbing on their legs when getting up from the floor (figure1).

Figure 1. Gowers manoeuvre

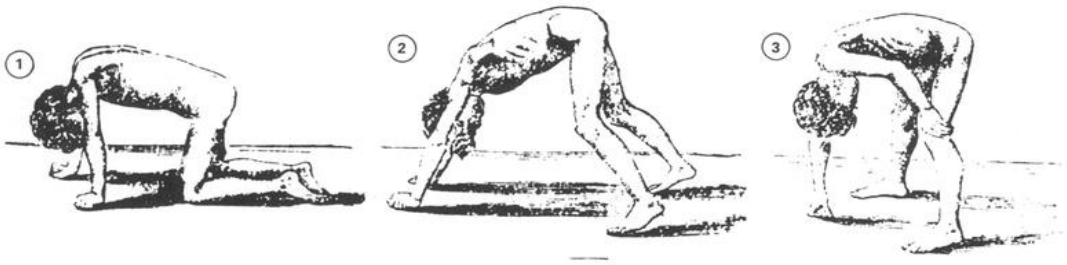


Figure 1. Gowers manoeuvre *(Gowers WR. Clinical lecture on pseudohypertrophic muscular paralysis. Lancet 1879;ii,73-5.)*

However, children may be symptomatic under three years of age, even if signs are subtle, showing gross motor and/or language delay when compared to their peers.[11-13] Recent studies suggests that while there are gains in motor skills in the early years, when compared to their peers, even infants and young boys with DMD may be losing ground and show less maturational improvement with age.[11-13] In addition, many boys with DMD have social deficits, which are generally appreciated at school age. However it has been demonstrated on objective clinical evaluator assessments, those DMD boys younger than three show lower average performance on measures of cognitive and social emotional development.

The diagnosis can be suspected following abnormal laboratory result: such as elevated creatine kinase (CK), or transaminases (ALT/AST), discovered incidentally during investigations for other on-going illness. Table 1 summarises various presentations of DMD.

Table 1. Various presentations of DMD

Motor presentations	
	Walking delayed beyond 18 months
	Frequent falls
	Toe walking
	Foot posture deformities
	Waddling gait
	Difficulty running, jumping and hopping
	Difficulties rising from the floor
Non-motor presentation	
	Global developmental delay
	Severe intellectual disability
	Neurodevelopmental disorders
	Failure to thrive
	Myoglobinuria
	Malignant hyperthermia-like reaction to general anaesthetic
	Incidental elevated CK
	Incidental elevated transaminases (ALT/AST)
	Positive family history

Diagnosis

a. Creatine Kinase

Serum creatine kinase (CK) is extremely elevated, 10 to 100 times the upper limit of normal (normal levels 2-251 U/L) since birth, and should be the first investigation when DMD is suspected. These CK values may fall to much lower levels during the later stages of the condition, as a result to muscle wasting and decreased muscle activity. A normal CK at presentation generally excludes the diagnosis, however maybe an exceptional finding in patients with X-Linked dilated cardiomyopathy or Becker Muscular dystrophy,[14] which are the mildest end of the dystrophinopathies spectrum. Other liver enzymes, such as aspartate transaminase (AST) and alanine transaminase (ALT) can also be 10 to 20 times above the upper limit of normal (ALT normal levels 5-30 U/L; AST normal levels 0-41 U/L) due to their production also in skeletal muscle.

c. Molecular genetics

Confirmation of diagnosis by molecular genetics provides the clinical information for genetic counselling, prenatal diagnosis and considerations for mutation specific therapies/clinical trials. Approximately two-thirds of the affected patients have large deletions in two mutational hot spots of the gene, which can be detected by multiplex PCR. [15] For more in-depth analysis Multiple Ligation dependant Probe Amplification (MLPA)[16, 17] and multiplex amplifiable probe hybridization,[16] which recently have become more available, will cover all exons and detect all deletions and duplications. If MLPA testing is negative, and diagnosis highly suspected, *DMD* gene sequencing should be

performed to look for point mutations, or small deletions/insertions, which are found in about 13% of DMD patients.[18]

Of note, the different types of mutations, such as missense, nonsense, splice-site mutations, single base deletions, larger deletions and duplications or rarer intronic rearrangements, are spread across the whole *DMD* gene.[19, 20]

Advances in molecular genetics now also allow precise evaluation of the carrier status of the relevant females in a family.

d. Histopathology

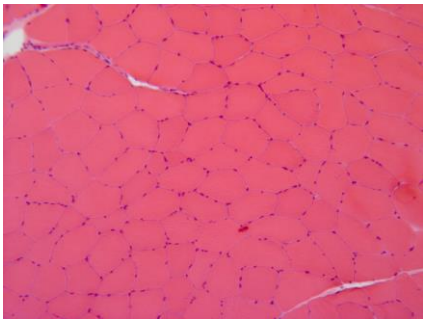
A muscle biopsy is not necessary if a genetic diagnosis has been established.[21] Nevertheless, the correlation between genotype and phenotype is approximately 90%[19] and therefore if a more precise correlation is required especially for patients with mutations that have been associated with different phenotypes, a muscle biopsy may be indicated in these cases for a more firm diagnosis.

For example, some patients do not follow the reading frame rule, therefore out-of frame mutations predicted to be associated with a DMD phenotype may present with an intermediate/Becker phenotype, and vice-versa, in-frame mutations can be associated with DMD in up to 7% of cases.[22, 23] In such circumstances, a muscle biopsy can elucidate if residual protein is expressed, resulting in a milder phenotype.

Tests performed on muscle biopsy include immunohistochemistry and immunoblotting of dystrophin.

Figure 2. Muscle biopsy images

a)



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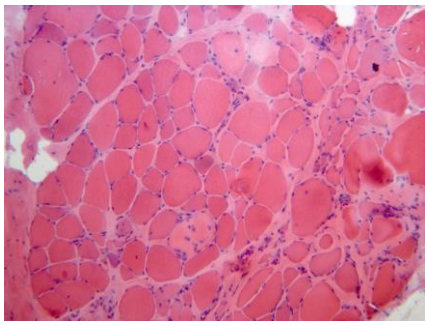

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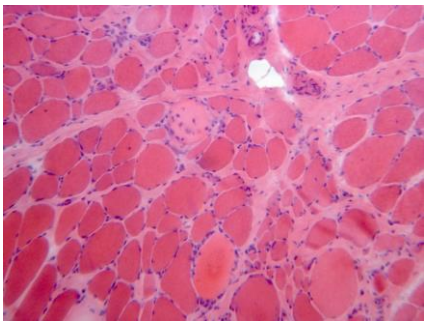

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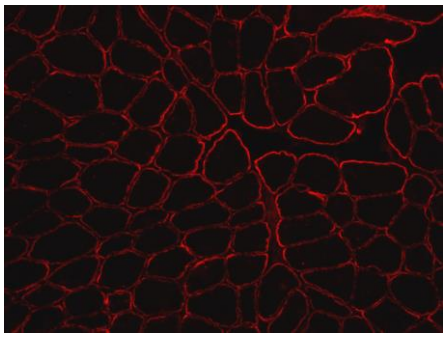

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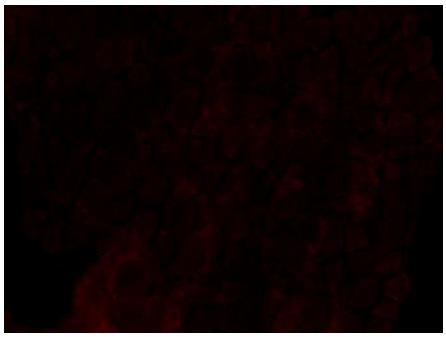
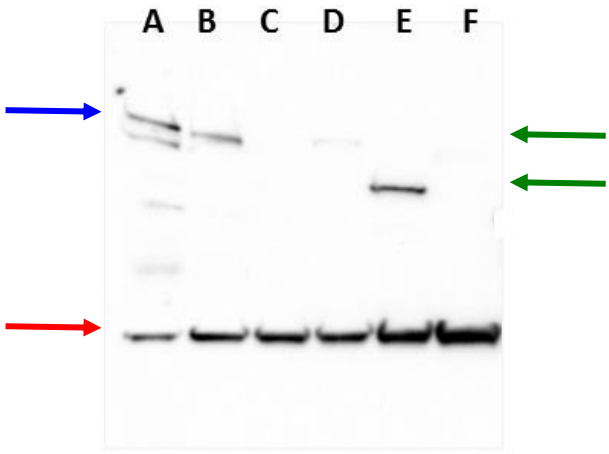




f)

Figure 2. H&E staining of a normal muscle (a). H&E staining of a young DMD boy showing fibrosis, necrotic and basophilic fibres (b), fat infiltration, hypertrophic and atrophic fibres (c). Dystrophin immunohistochemistry: normal dys 3 labelling in control (d) and absent dys 3 labelling in DMD (e). Western blot-sarcomeric α-actinin (f): normal control (A), DMD subjects (C,F) and BMD subjects (B,E). Red arrow: α-actinin; blue arrow: normal dystrophin, green arrows: dystrophin of reduced molecular weight in BMD.

(Curtsey of Lucy Feng and Silvia Torelli, Dubowitz Neuromuscular centre, UCL Institute of Child Health London.)

Dystrophin is a rod-shaped protein localised at the inner side of the sarcolemma and is part of a complex of (glycol-)proteins: the dystrophin-associated protein complex (DAPC) (figure 3). Many muscle proteins, such as α-dystrobrevin, syncoilin, synemin, sarcoglycans, dystroglycan, and sarcospan, colocalize with dystrophin at the costamere and are believed to share signal pathways. The role of dystrophin is yet not entirely understood, however it is undisputable that it is a key protein in the striated muscles, which offers a mechanical support and stability to the muscle fibre. When dystrophin is disrupted this leads to an increase in muscle fibre fragility, in part due to the loss of mechanical connection between the sarcolemma and the actin cytoskeleton, but possibly also secondary to a disturbance in calcium homeostasis resulting in fibre degeneration. [24] Furthermore, it is now recognised that DMD patients because of the lack of dystrophin which harbours a nNOS binding site fail to localise nNOS to the sarcolemma. This leads to nNOS mis-localization the cytosol and to pathological nitrosylation of the ryanodine receptor resulting in increased calcium leakage into the cytosol.[25] In addition, lack of functional nNOS at the sarcolemma adds to an abnormal regulation of blood flow within exercising muscles.[26] Much remains unknown, however, of the different factors contributing to the specific clinical pattern of the disease.

Figure 3. Dystrophin-glycoprotein complex.

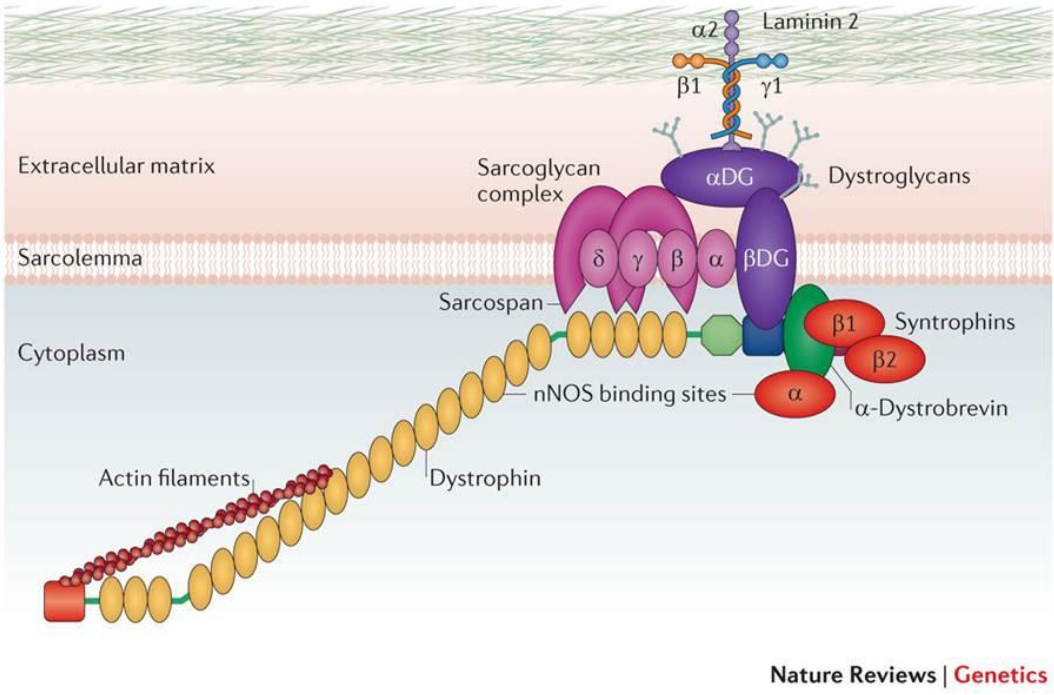


Figure 3. The dystrophin-associated protein complex. *(Source: Fairclough, Wood and Davies, Nature Genetics 2013.)* Dystrophin is a rod shape protein that links intracellular cytoskeleton network to transmembrane components, which include dystroglycan, sarcoglycans, and sarcospan. Neuronal nitric oxide synthase (nNOS) binds to alpha-syntrophin and in the rod domain of the protein.

Progression

Following an initial period of apparent stability in relation to muscle weakness, as they become older, DMD boys become progressively weak. Muscle weakness is more pronounced in the hip extensors, as a result the pelvis becomes unstable. Contractures also develop, most commonly affecting the Achilles tendons (TA) and the iliotibial bands (ITB). The disease evolves into muscle atrophy, and loss of ambulation, historically between 6-12 years of age. With the progressive weakness affecting also the upper body, DMD patients will develop progressive

scoliosis, secondary to weakness of the para-spinal muscles, which also affect respiratory function. Before ventilation was introduced in the late 1970s, death occurred in the late teens or early twenties in 90% of patients secondary to respiratory complications, and 10% of patients secondary to dilated cardiomyopathy (figure 4).[27 28]

Management

Despite the major advances in understanding the underlying pathogenic mechanisms of the disease, no cure is currently available for DMD. However, life expectancy for DMD patients has now shifted well into adulthood with reported age of death in the 30s-40s,[29-31] as a result of the implementation of internationally agreed standardised guidelines of clinical care.[32, 33] The standards of care for DMD encompass a multidisciplinary medical, surgical and rehabilitation approach of the multiple symptoms of the disease. The key interventions, which have profoundly altered the disease course, include respiratory support, surgery for scoliosis,[31] physiotherapy and intervention with glucocorticoids.[34, 35]

The fundamental pillars of the multidisciplinary approach are summarised in the table below:

1) **Orthopaedic management and rehabilitation**
    a. Preventive measures for the progressive contractures include mobilization, stretching and ankle-foot orthoses mostly worn at night-time.[36, 37]
    b. Prolongation of ambulation for 6-8 months can also be achieved with knee-ankle-foot-orthoses.[38]

c. Regular clinical surveillance of scoliosis is imperative;[39] and an anterio-posterior radiograph is warranted if the curvature is >15^{o}. A multidisciplinary team approach is required in establishing suitability for scoliosis surgery in patients who are non-ambulant and the curvature is >20^{o}. Fortunately, glucocorticoid therapy has considerably reduced incidence of scoliosis, hence the need for surgical intervention.[40, 41]

2) Glucocorticoid therapy

The use of chronic glucocorticoid therapy in the ambulant phase is endorsed by the standards of care documents, and supported by an extensive Cochrane review.[42] There is evidence that steroids improve muscle strength,[42, 43] prolong ambulation and function,[35, 43] prevent scoliosis[41, 44] and delay the onset of respiratory failure[43-45] and possibly that of cardiac dysfunction.[44, 46, 47] Steroid therapy, including regimens, doses and side effects will be further discussed in the following chapter.

3) Respiratory Management

Guidelines for the respiratory management include the use of cough assist and timely intervention with non-invasive ventilation.[48] Surveillance of respiratory function with spirometry and with sleep studies when required is imperative.[48] Since the introduction of ventilation alone, life expectancy has shifted to 30 years.[29] Furthermore, immunization with 23-valent pneumococcal polysaccharide vaccine and annual immunization with inactivated influenza vaccine is indicated.[33]

4) Cardiac Management

Lack of dystrophin in the myocardium invariably leads to a subclinical cardiomyopathy, which develops into a progressive dilated cardiomyopathy and/or arrhythmia from the second decade onwards. [49] Cardiomyopathy is the leading cause of death in DMD. Regular surveillance of cardiac function is essential. Current management includes symptomatic treatment with angiotensin-converting-enzyme inhibitors (ACE-I), and if required beta-blockers and diuretics.[50] A recent US study showed significant benefit when an aldosterone antagonist, eplerenone, as an adjuvant therapy.[51] Encouraging results from Phase I and Phase II studies support treatment with ACE-I for the prevention of cardiomyopathy. [52] Furthermore, a retrospective study showed that the combination of an ACE inhibitor and a beta-blocker had a beneficial effect on long-term survival of DMD patient, suggesting a potential role also for prevention. [53] A Phase III randomised placebo-controlled multicentre clinical trial is currently on-going in the UK to establish efficacy of preventative therapy.

5) Endocrinological management

Endocrinological aspects have been increasingly gathering the attention of clinicians especially to address glucocorticoid therapy associated side effects.[54]

a. Promoting bone health and preventing steroid induced osteopenia is imperative. Guidelines recommend close surveillance of bone density with yearly DEXA scans while on steroids, and

monitoring for vertebral compression fractions.[55-57] Furthermore it is recommended to keep the levels of 25-OH-vitamin D within normal ranges with chronic prophylactic supplementation. [57]

b. Delayed puberty is invariably observed in steroid treated patients. Induction of puberty with testosterone injections is often practised, however there is no published data on the long-term outcomes.[54]

c. Short stature secondary to steroid-induced reduced growth velocity is well recognised. Therapeutic options include growth hormone IGF-1, however the evidence of IGF-1 as a beneficial therapy remains sparse,[58] whilst a short stature can possibly provide advantage to motor function.[59]

d. Finally, there is increased awareness of steroid-induced glucose intolerance. Metformin, an insulin sensitizing agent, can be considered in selected cases.[54]

6) Nutrition

a. The major concern with decreased mobility and steroids is excessive weight gain. Individualised dietary advice on caloric intake and appetite control is very important.[54]

b. Decreased function of visceral smooth muscle is recognised in DMD.[60-62] When symptoms of constipation are present many patients use iso-osmotic laxatives to facilitate transit. With the progression of the disorder pharmacological intervention may be required to manage constipation and gastro-oesophageal reflux.

Gastrostomy tube may be placed to prevent aspiration and optimise nutrition.[33]

7) **Exercise**

Promoting exercise in DMD has been controversial. It is widely recognised that high resistive eccentric contractions are damaging for the dystrophic muscle fibres, whilst beneficial effects have been reported for low-intensity training.[63] However, further research is needed to establish the optimal exercise training, which does not confer detrimental effects on the muscle in the long term as well as on the heart.

8) **Psychosocial management**

The medical care of DMD would not be complete without the appropriate psychosocial support. Psychosocial adjustment at diagnosis and during disease progression require careful considerations.[64, 65] Furthermore, there is a growing body of literature detailing the cognitive impairment and neuropsychiatric comorbidities of DMD, which require prompt interventions. [66-68] [69-71] [70, 72, 73]

Evolving natural history and novel frontiers of therapy

Undoubtedly the natural history of Duchenne muscular dystrophy has considerably evolved in the past decades, with a mean age of loss of ambulation in the mid-teens and life expectancy reaching mid-40s.[29, 32, 33] A schematic representation on its evolution is illustrated in figure 4. The natural history of DMD continues to be a moving target. With the implementation of the standards

of care and the rapidly growing attention to the multifaceted aspects of the disease, life expectancy will shift even further and quality of life is also likely to improve.

In the past decades a number of promising potential therapeutic approaches have been developed, some have now reached phase II-III clinical trial trials[74-82]. With the emergence of novel therapies there has also been a need to develop and sharpen skills for the design of clinical trials, especially challenging for a rare and progressive disorder such as DMD. Moreover, these novel experimental therapies will further contribute to the transformation of the disease trajectory and will generate yet new questions to be addressed also as part of the standards of care.

The projects of my thesis intend to address specific research questions, which have an impact not only on the clinical care of DMD patients, but also advise on the clinical trials design.

In **Chapter 2** I will discuss the impact that steroid therapy had on the motor function in DMD boys over 7 years of age, more specifically profiling benefits and side effects of the most commonly used regimens, intermittent 10 days on and days off and daily prednisolone.

In **Chapter 3** I will report the impact of starting steroids at an earlier age than what has been generally practiced in clinic. Furthermore I will discuss the role that genotypes play in the progression of the disease, further defining the genotype-phenotype associations. This is particularly of interest for the clinical trials of emerging pharmaco-gene or gene therapies.

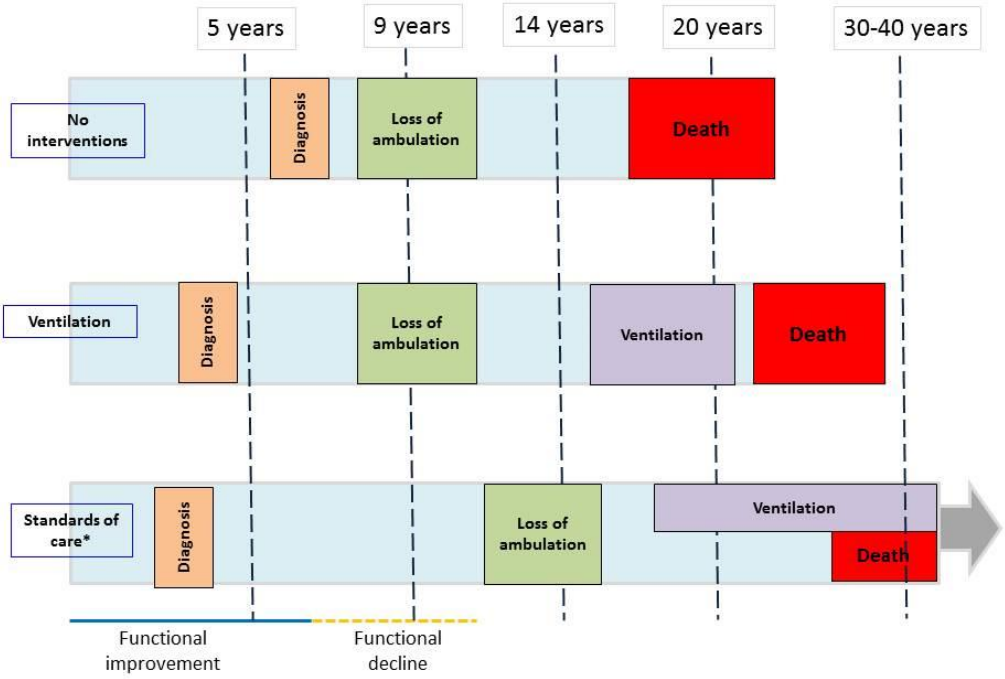
In **Chapter 4** I will discuss the role of dystrophin and its different isoforms in the central nervous system (CNS), more specifically profiling the neuropsychiatric co-morbidities in DMD.

In **Chapter 5** I will expand on the CNS role of dystrophin more specifically related to retinal function and discuss the potential use of electroretinography (ERG) as an explorative endpoint in clinical trials

In **Chapter 6** I will discuss magnetic resonance imaging (MRI) and novel upper limb outcome measures in the non-ambulant population. Assessments of the upper-limb in patients who are no longer ambulant offer the opportunity to monitor disease progression beyond the loss of ambulation. Furthermore MRI offers an alternative and non-invasive method to monitor disease progression and therapeutic response in a context of clinical trials.

In **the final Chapter,** I will discuss the challenges of clinical trials design and potential future avenues.

Figure 4. Schematic representation of the natural history of DMD



(Adapted from Bushby & Connor, Clin. Invest. 2011)

Figure 4. Evolution of the natural history of DMD from no intervention, to the introduction of the standards of care, which include steroid therapy, ventilation, physiotherapy, orthotics and spinal surgery. * *Bushby et al. Lancet Neurol 2010 (1 &2)*

Notes on the history of the disease

The disorder has been eponymically attributed to the renowned French Neurologist Guillaume Benjamin Amand Duchenne (1803-1875). In 1858 he studied the case of a 9-year-old boy who could not walk due to muscle wasting. In his seminal publications of 1861 and 1868 and his second edition of *De L'Electrisation localisee* [83] Duchenne describes the disease in considerable detail, which crystallises all the essential features including the histopathology findings

which he obtained by use of his invention, the "harpoon" to perform percutaneous muscle biopsies. In his publication, he proposed the name of "hypertrophic paraplegia of infancy" based on the presenting symptoms. He described the male predominance, waddling gait, the progressive course, loss of ambulation, pseudo-hypertrophy of the calves, and on muscle biopsy he described the loss of muscle fibre striation, hyperplasia of fibrous connective tissues, fat infiltration, and fascicular atrophy.

However, the first historical account of DMD was reported by the Neapolitan physician Giovanni Semmola in 1834 and Gaetano Conte in 1836.[84] They described 2 brothers with progressive weakness starting at age 8 years and enlarged calves, who subsequently developed contractures. The older brother died of cardiac failure. At the time, it was thought that this clinical profile was perhaps due to tuberculosis of the muscles.

About 10 years before Duchenne, Edward Meryon, an English physician, had described at the Royal-Medico-Chirurgical Society a family of four affected brothers and six healthy sisters. Meryon was the first to carry out a systemic clinical family and pathological study of the disease. He was the first to recognise that this condition was a primary disease of muscles; subsequently on autopsy he described the granular degeneration of the tissue, and disruption of the sarcolemma, whilst the spinal cord was intact, and he showed that the disease was familial, with boys the only affected.[85, 86]

In his own works, Duchenne refers on several occasions to the papers published by Meryon, however he raised concerns that Meryon had mistakenly described

his cases as progressive muscular atrophy.[83] This misconception about Meryon's description was fuelled by other renowned physicians, such as Lockhart Clarke, who was in close contact with Duchenne and co-authored with Gowers a case of a young boy identified to have the "pseudo-hypertrophic paralysis" described by Duchenne, and at autopsy degeneration of nerve-cells in the grey matter, which was in contrast with the normal findings of Meryon.[87]

The term "pseudo-hypertrophic dystrophy" was established when William Gowers, an English neurologist, gave a series of lectures to the students of University College Hospital describing 21 personal cases and 139 cases previously described in the literature.[88] These lectures where subsequently published in the Lancet.[88] William Gowers also described the classical manoeuvre by which affected children get up from the floor.[88]

Since then, important milestones in the history of DMD include the identification of elevated serum creatine kinase (CK) in affected individuals and female carriers;[89] mapping of the dystrophin gene to Xp21;[90] the identification localization and functional role of the protein dystrophin;[91-93] and finally the recognition and description of Becker muscular dystrophy as the allelic and less severe variant to DMD.[94]

CHAPTER 2: STEROID THERAPY IN DMD

The work contained in this chapter has given rise to the following publication: **Ricotti V**, Ridout D, Scott E, Quinlivan R, Robb S, Manzur AY, Muntoni F, on behalf of NorthStar Clinical Network.
Long term benefits and adverse effects of intermittent versus daily glucocorticoids in boys with Duchenne Muscular Dystrophy. J Neurol Neurosurg Psychiatry. 2013 Jun;84(6):698-705. PMID: 23250964.

My contribution to this work consisted of the following: I oversaw the design and conduct of the study, and analysis. I oversaw and contributed to data collection. I supervised and organised the inclusion of patients from Great Ormond Street Hospital into the database. I led the analysis of the data in collaboration with the statistical department at UCL, ICH. I wrote the first draft of the manuscript, and I contributed to the revision of the manuscript.

Glucocorticoids in DMD: Setting the scene

Glucocorticoids (GC) have been used in DMD for about four decades. As early as 1974, Dracham *et al.* observed in an uncontrolled study on 14 DMD patients aged between 3 and 10 years that GC had an effect in slowing progression of the disease.[95] This observation, however, was not supported by a controlled study conducted by Siegel *et al.* over 24 months in a small cohort of 7 Duchenne boys aged 6-9 years compared with 7 matched controls. The authors reported no value in GC therapy.[96]

Despite Drachman's promising initial observation, no further systematic studies were conducted until much later. In 1987 Brooke *et al.* set up the first multicentre open study of 33 DMD patients aged between 5 and 15 years. The authors reported overall improvement when compared to natural history controls with a

dose of 1.5 mg/kg; muscle strength, joint contractures, timed functional tests, functional ability, and pulmonary function were measured at the beginning and 6 months later at the end of the treatment period.[97, 98] At the same time, De Silva reported long-term benefits in 20 DMD boys in relation to age of loss of ambulation, when compared to historical controls.[99]

The first randomised controlled trial was a US multicentre study including 103 subjects aged 5 to 15 years over 6 months, comparing two different regimens of prednisone 0.75 mg/kg/day and 1.5 mg/kg/day vs placebo.[100] The authors reported that improvement was observed as early as one month and peaked by three months. At six months the high-dose group (i.e. 1.5 mg/kg/day) when compared to the placebo group showed improvement ($p<0.001$) in the time rising from the floor from supine (3.4 and 6.2 sec respectively), the time to walk 9 m (7.0 and 9.7 sec respectively), in the time to climb four stairs (4.0 and. 7.1 sec respectively), in strength measured as lifting a weight (2.1 and 1.2 kg respectively), and in respiratory function measured as forced vital capacity (1.7 and 1.5 litres respectively). The most frequent side effects observed during the study included: excessive weight gain, Cushingoid appearance, and excessive hair growth. Given the profile of side effects observed in a short time frame of therapy, the authors concluded that GC could not be recommended for long-term palliative therapy until studies assessing prolonged treatment were carried out.[101]

The same group of patients was further evaluated to compare alternate-day and daily dosing of prednisone with respect to benefits and adverse effects. [102] The placebo arm was started on alternate-day prednisone therapy, and the treatment group regimens were changed to equivalent doses of alternate-day prednisone. At the end of 6 months, the group that was changed from daily to alternate-day

GC had declined in strength back to levels observed at baseline. The group in which alternate-day therapy was started showed a significant improvement in strength at 3 months, not dissimilar to the response of boys treated with daily therapy. However, their strength declined significantly afterwards. The frequency of side effects was not significantly different for alternate-day therapy compared with daily therapy. The authors concluded that alternate-day prednisone therapy effectively increases strength but does not sustain the improvement to the same extent as daily therapy nor does it mitigate side effects.[102]

The same research group carried out a study to establish the optimal therapeutic dose of prednisone by comparing 0.75 mg/kg/day and the lower dose 0.3mg/kg/day *vs* placebo over 6 months in a cohort of 99 patients aged 5-15 years. [103] At the 3-month visit, the boys in the group receiving 0.75 mg/kg of prednisone were stronger than those in the group given 0.3 mg/kg of prednisone. At 6 months, more severe side effects occurred in the group treated with the higher dose, whilst excessive weight gain was the only side effect reported in the group treated at the lower dose of 0.3 mg/kg. [103]

These results were the first to establish a therapeutic window for DMD. Moreover, Fenichel *et al.* reported about the benefits of GC therapy after 3 years of treatment. The same cohort of boys earlier recruited in the 6-month randomised double-blind controlled trials (n=93) were treated for two additional years. At the time of their last visit most of the boys in addition to maintaining their strength and their performance in lifting kilogram weights, had also improved in some timed function tests. [104]

In an editorial published in 1991 Dubowitz[105] commented the recent studies on steroids and recommended an intermittent regimen as a potential avenue to avoid the side-effects of prolonged GC therapy (Table 2). Given the clinical rapid improvement in muscle strength within 10 days even on low dosage, Dubowitz proposed short repeated periods of low dosage daily steroids, with intervals of no treatment in between. The periods of treatment would have to be short enough to avoid adrenal suppression and associated difficulty in withdrawing the drug. One simple regime suggested in the editorial was prednisone 0.75 mg/kg/day for 10 days at the beginning of each calendar month, followed by no treatment for the remaining 20 days of the calendar month.

Table 2. Adverse effects associated with chronic systemic glucocorticoid use

Metabolic/endocrinologic effects	• Hypokalemia • Hyperglycemia • Hyperlipidemia • Adrenal Suppression • Growth Suppression • Delayed Puberty • Excessive Weight gain • Diabetes mellitus • Cushingoid Habitus
Musculoskeletal effects	• Osteoporosis • Vertebral compression fractures • Aseptic necrosis of bone • Myopathy
Dermatologic effects	• Dermal thinning and striae • Increased skin fragility • Acne • Hirsutism
Ophthalmological effects	• Cataracts • Glaucoma
Immunologic effects	• Diminishing IgG levels • Loss of delayed-type hypersensitivity • Potential for increased risk of opportunistic infection, reactivation of latent TB, or severe varicella infection
Haematological effects	• Lymphopenia • Neutrophilia
Cardiovascular effects	• Hypertension • Atherosclerosis
Psychological/neurologic effects	• Mood swings • Steroid withdrawal syndrome • Idiopathic intracranial hypertension • Psychosis

Table 2: Adverse effects associated with chronic systemic glucocorticoid use as reported in Nelson Textbook of Paediatrics, 17th Edition (*Saunders editors*).

An open trial was conducted on 32 DMD subjects close to loss of ambulation, and at 6 months demonstrated that this regimen had a positive effect on some individuals when strength was measured, however this was followed by a slow decline at 12 and 18 months. [106] Weight gain and other side effects observed with a continuous regimen were less severe in this group of patients.[106]

In 2005, Beenaker et al. designed a study that focused on the initial proposed intermittent regimen.[107] The authors studied 17 ambulant DMD patients aged 5-8 years in a randomized, placebo-controlled, crossover trial with 6 months of treatment: prednisone or placebo (0.75 mg/kg daily) during the first 10 days of each month. After a washout period of 2 months, patients received the other regimen for an additional 6 months. Timed functional testing were the primary outcome measures, which showed significant difference from placebo; this included the time-required-to-run-9-metres (p=0.005) and to climb 4 standard-sized stairs (p=0.02). The authors concluded that intermittent prednisone slowed deterioration of muscle function and muscle force in ambulant patients with DMD. [107] Subsequent to the initial suggestion of 10 days-on and 20 days-off therapy, in order to increase efficacy such intermittent regimen was modified to give prednisolone 0.75 mg/kg/day 10 days on and 10 days off. Encouraging long-term results were presented on a case series of 4 boys.[108, 109]

An alternative intermitted regimen has been proposed by Connoly *et al*.: twice weekly oral prednisone was given each Friday and Saturday given as 5mg/kg/dose.[110] A cohort study was reported in 2002 suggesting improvement in grip strength in 20 treated boys at 12 months, when compared with historically untreated cohorts; however growth and weight did not differ from the historical controls, suggesting less side effects.

Furthermore, deflazacort has also been proposed as an alternative to prednisolone/prednisone. One randomised controlled trial comparing deflazacort to prednisolone has been published by Bonifati in 2000; [111] the study included 18 patients and compared daily regimens of prednisolone and deflazacort. The outcomes showed similar benefits over one year; however the boys treated with deflazcort did not show the same degree of excessive weight gain.

The complete list of studies on GC in DMD up to 2008 is reported in the updated Cochrane Review: Glucocorticoid corticosteroids for Duchenne muscular Dystrophy.[34] The authors concluded that the most effective regimen appears to be prednisolone 0.75mg/kg/day or the equivalent Deflazacort 0.9 mg/kg/day. However, on published data, the authors could not evaluate long-term effects of GC and different regimens, given the short term nature of the published studies. The main limitations of the studies appraised by the Cochrane Review relate to the number of patients evaluated, the duration of the studies and the selection of outcomes measures.

With the exception of the studies reported by Mendell et al.[101] and Fenichel et al. [102], which included 103 DMD patients (two daily doses *vs* placebo)[101] and subsequently followed-up 102 boys (daily vs alternate days), [102] most of the early studies included between 15-60 patients. Griggs *et al.*[103] studied a total of 99 DMD boys, however only 70 of those were ambulant, and in the same study different doses were evaluated. With the current knowledge, it is recognised that response to therapy varies between subjects, and also according to the stage of the disease; therefore a sample size of even 100 patients spanning from 5-15 years of age, from ambulant to non-ambulant and evaluating different doses/regimens, is largely underpowered. Furthermore, the duration of these

studies was relatively short. Only 2 randomised studies were conducted for 24 months. [96, 112] The study reported by Siegel *et al*. [96] however only evaluated 7 DMD boys, hence not surprisingly the 2 year results were inconclusive. Pradhan *et al*. [112] also conducted a study over 2 years, however targeting a DMD population in a more advanced stage and close to losing ambulation (mean age 8.5 years). Despite the general consensus that a response to steroids can be clinically observed as early as 3 months, in order to evaluate long-term effects and side effects, short-duration studies are not adequate. Moreover, none of these early studies were immune to selection bias, and the authors did not address this point. Furthermore, most of these early studies did not evaluate response to treatment with validated functional scales or outcome measures (e.g. the NorthStar ambulatory assessment), but relied on manual muscle strength measurements, which can be operator-dependant, or timed tests (i.e. time taken to rise from the floor), which can be motivation-dependant. Finally, the intermittent regimen 10 days on/off suggested by Dubowitz[105] and now widely used in the UK was only evaluated on 4 DMD boys by Kinali *et al*.[108]

Most recent work include the study by Mazzone et al.[113], which evaluated functional changes in a national cohort of 106 steroid treated patients using validated outcome measures (e.g. 6-minute walk test and NSAA) as primary endpoints. [113] Furthermore, a recent randomised-blinded clinical trial compared high dose weekend prednisone (10 mg/kg/week) vs standard daily dose (0.75 mg/kg/d) over 12 months in 64 DMD boys.[114, 115] The study provided evidence measuring both force and function that high dose weekend prednisone is equally effective in preserving muscle strength/function, while the side effect profile was similar in the two regimes.

A study published in 2015 by Bello *et al*. for the first time reported on the long-term effects of deflazacort therapy in 80 DMD patients, showing that the use of daily deflazacort was associated with prolonged ambulation when compared to any prednisolone regimen; however Cushingoid features, growth retardation and cataracts were more frequent.[116]

Finally, a very recent study for the first time systemically evaluated upper-limb function in steroid treated non-ambulant DMD boys, supporting the current consensus among clinicians that GC should not be discontinued after loss of ambulation.[117]

Table 3 summarises the key prospective studies that evaluated GC therapy.

Table 3. Prospective published studies on prednisolone treatment: design, sample size glucocorticoids regimen and duration of treatment.

STUDY	GC regimen	Design	Duration	Number of patients
Mendell 1989	Daily	Randomised double blinded	6 months	103
Fenichel 1991a	Alternate days/daily	Randomised	6 months	102
Griggs 1993	Daily	Randomised	18 months	99
Bonifati 2000	Daily	Randomised	12 months	18
Pradhan 2006	Daily	Randomised	24 months	67
Rahman 2001	Daily	Randomised	6 months	19
Griggs 1991	Daily	Randomised double blinded	6 months	99

Drachman 1974	Alternate days	Open	3- 28 months	14
Siegel 1974	Alternate days	Double-blind	24 months	14
Brooke 1987	Daily	Open	6 months	33
DeSilva 1987	Daily first then alternate daily	Open	1-11 months	16
Beenakker 2005	Intermittent	Randomised double blinded	6 months	17
Backman 1995	Daily	Randomised double blinded	6 months	41
Parriera 2007	Daily/ Intermittent	Cohort	14 months	32
Yilmaz 2004	Alternate days	Cohort	3 years	66
Fenichel 1991b	Daily	Open	2 years	93
Sansome 1993	Intermittent	Open	6-18 months	32
Kinali 2002	Intermittent	Open	5 years	4
Connolly 2002	Weekend high dose	Open	22 months	20
Mazzone 2011	Intermittent and daily	Cohort	12 months	106
Escolar 2011	Daily vs weekend high dose	Randomised blinded	12 months	64
Bello 2015	14 different regimens	Cohort	4 years	340
Pane 2015	Daily/ Intermittent	Cohort	12 months	91

Mechanism of action of glucocorticoids

The precise mechanism of action of GC in dystrophic muscles remains largely poorly understood. In unaffected subjects the most prominent change in skeletal muscle following GC treatment is a mixed atrophy affecting type II fibres more severely than type I fibres.[118] Studies have consistently shown that steroids increase number of mitochondria in both sub-sarcolemmal and inter-myofibrillar loci, and this alteration is expected to be beneficial in dystrophies, since mitochondrial increase should confer prolonged activity and fatigue resistance.[119] The precise mechanism of their action is unknown, but prednisolone- treated DMD muscle has shown a significant anti-inflammatory effect. Mononuclear cell analysis showed significantly fewer T cells, cytotoxic/suppressor T cells and less muscle fibres focally invaded by inflammatory cells, compared with muscle biopsies from the placebo group.[120]

In addition it has been shown that prednisolone enhances myogenesis in dystrophin-deficient skeletal muscle of the dystrophic animal model: the *mdx* mouse. It has been hypothesized that GC reduce muscle necrosis and stabilise the muscle fibre membrane.[121] In *mdx* dystrophic mice, RNA profiling studies identified over-expression of metabolism, proteolysis and structural protein genes. [121] And finally, Fischer *et al.* demonstrated that skeletal muscle gene expression profiles of mdx mice, treated with prednisolone when compared with control mice at 1 and 6 weeks showed a differential expression of calcium metabolism genes.[122] At both time points, overexpression of a cohort of genes relating to metabolism and proteolysis was apparent, alongside the differential expression of genes relating to calcium metabolism. In the treated *mdx* soleus muscle, the percentage of slow fibres was significantly lower compared with untreated controls after 6 week of treatment.

All of these results show how complex the GC action is upon dystrophic muscle, and how many different pathways are likely involved in conferring benefit to dystrophin-deficient tissue.

Note on glucocorticoids

Prednisone is an ester pro-drug that is converted to the active prednisolone in the liver through the action of 11-beta-hydroxydehydrogenase. Both prednisone and prednisolone have equipotent GC effect. Deflazacort is a methyloxazoline derivate of prednisolone: 1.2 mg of Deflazacort is equivalent to 1 mg of prednisolone.

A study on long term benefits and adverse effects of intermittent versus daily glucocorticoids in boys with Duchenne muscular dystrophy

In my study I analysed data collected through the NorthStar clinical network for paediatric neuromuscular disease (NSCN), from January 2004 to September 2011. We focused on clinical outcomes from different GC regimens and analysed outcome measures and side effects in the largest steroid-treated cohort of DMD boys studied to date.

Methods

The NorthStar Clinical Network and database

The NSCN was established in the United Kingdom at the end of 2003, with the objective of optimising the care and acquiring longitudinal natural history data

on DMD boys treated and assessed according to a specified standardised protocol, including GC.[32, 33] Currently there are 21 participating specialist paediatric neuromuscular centres. A full list of participating centres is available on line http://www.northstardmd.com/study-centres.html and in the appendix. A secure web-based database has been set up with the collaboration of CERTUS technology, and is currently used for data collection since 2006. Prospective data are uniformly and systematically collected across centres facilitating national audits. Physicians and physiotherapists complete standardised forms biannually. A national training programme was implemented to ensure standardisation of physiotherapy data collection across centres and a national coordinator assures standardised re-training across the network. Baseline information is recorded at registration (table 4). At each follow-up, medical and physiotherapy data are documented including steroid regimen, side effects, outcome measures, and a management plan (table 4).

The NorthStar database collects clinical information on DMD boys with an out-of-frame mutation in the DMD gene, confirmed by DNA diagnostic technique covering all DMD gene exons, including but not limited to Multiplex Ligation-dependent Probe Amplification. Where DMD deletions or duplications were not identified, all 79 exons and the adjacent introns were analysed through PCR amplification and direct sequencing, although the search for point mutation was not available uniformly throughout UK. Mutations are classified according to the Leiden Muscular Dystrophy database.[123]

Boys with no confirmed mutation but absent dystrophin in muscle biopsy (<5% on immunohistochemistry) are also included in the database. Information is recorded with signed parent/guardian informed consent. The data is linked-anonymised and each subject is assigned a unique NorthStar ID number.

Table 4. Key medical and physiotherapy information recorded on the database

Baseline information	Medical information at follow-up	Outcome measures at follow-up
Demographic data	Date of starting GC	Ambulation status and mobility aids
Genetic mutation§	Current dose and regimen of GC	Age at loss of independent ambulation
Maternal carrier status§	Adverse behavioural changes	Respiratory status (FVC, FVC%)
Date of diagnosis	Gastrointestinal symptoms	Echocardiogram (LVFS%)
Features of the muscle biopsy ǂ	Increased appetite	NorthStar Ambulatory Assessment score
Family and social history	History of infections	Time rising from the floor from lying
	Height and weight	Timed 10 metre run
	Blood pressure	Manual muscle testing
	Cushingoid features	Joint range
	Bone density measurements	Spinal posture
	Long bone fractures and vertebral fractures	
	Cataracts	
	Hirsutism	
	Delayed puberty	
	Other therapeutic interventions	
	Adjustment in GC dose/regimen	

Table 4: Key medical and physiotherapy information recorded on the database

§Genetic diagnosis and maternal carrier status confirmed by a state-of-the-art DNA diagnostic technique covering all DMD gene exons. ǂ Dystrophin expression observed on muscle biopsy by immunohistochemistry with monoclonal antibodies dys1, dys2, dys3 (i.e.: complete absence, traces).
GC = Glucocorticoid corticosteroids; LVSF% = Left Ventricular Shortening Fraction percentage; FVC% = Forced Vital Capacity percentage.

Patient population

At the time of my data analysis in September 2011, 500 patients were registered on the NorthStar database. Prospectively collected longitudinal data were available for 360 DMD boys between 2004-2011 (figure 5, table 5) ranging from age 3-17. Longitudinal data was available only for boys treated with GC, who represent the large majority of DMD boys in the age range studied. GC therapy is systematically offered and only exceptionally refused by parents. We classified boys according to the GC regimen: daily prednisolone (daily), intermittent prednisolone 10-days-on/10-days-off (intermittent), alternate days prednisolone, and deflazacort-treated. Some boys changed regimen of GC between daily and intermittent or vice-versa (switchers). All patients included were treated according to the integrated multidisciplinary standard of care,[32, 33] and assessed by specialised neuromuscular physiotherapists.

Figure 5. Flow-chart of boys included in the study

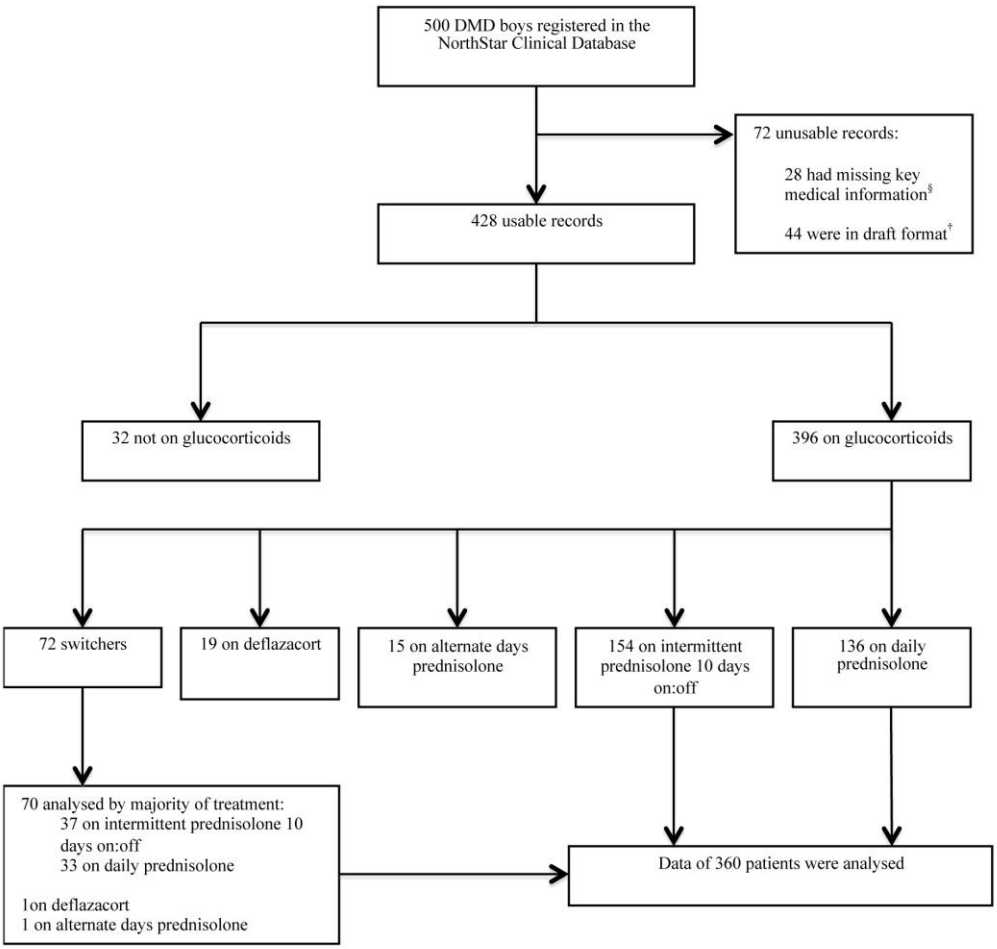


Figure 5: Flow of patients from registration in the database to enrolment in the study §Data with missing key medical information refers to essential data required for statistical analysis (e.g.: dose of glucocorticoids, date of birth of the patient, date of assessment).

†Assessments saved in draft format were not confirmed, therefore excluded from the analysis.

Table 5. NorthStar patients' breakdown by centre

Neuromuscular Centre	No. of patients	No of subjects recruited
Birmingham Heartlands Hospital	52	44
Frenchay Hospital, Bristol	11	2
University Hospital of Wales, Cardiff	14	14
Kings Cross Hospital, Dundee	9	6
Evelina Children's Hospital	16	14
Royal Hospital for Sick Children, Glasgow	9	0
Great Ormond Street Hospital, London	142	122
Leeds	35	26
Alder Hey Hospital, Liverpool	34	27
Royal Manchester Children's Hospital	36	24
International Centre for Life Newcastle upon Tyne	66	41
University Hospital, Nottingham	4	
Orthopaedic & District Hospital NHS Trust, Oswestry	24	16
Preston Royal Hospital	12	6
Sheffield Children's Hospital	19	15
Southampton General Hospital	15	3
Morriston Hospital, Swansea	2	0
Total	**500**	**360**

Table 5. NorthStar patients' breakdown by centre, completion percentage of data entry and number of patients recruited in the analysis as of September 2011. NorthStar Clinical Network Study centres are listed on the following website: http://www.northstardmd.com/study-centres.html.

Physiotherapy outcome measures

The NorthStar Ambulatory Assessment (NSAA) is a validated composite scale to measure function in ambulant DMD boys.[124, 125] It is widely used in the UK, internationally, and in clinical trials.[126] [79, 81] This scale has recently been confirmed by Rasch analysis to be a psychometrically robust scale.[127] The assessment consists of 17 items (table 6), with three ordered response categories (maximum score 34). Items can be scored 2 (activity carried out normally with no obvious modification), 1 (goal achieved independently with modified method), or 0 (task cannot be performed independently). Clear instructions are described in the data entry form. The scale is completed in 20 minutes and contains a number of timed tests: timed 10-metre-run-test (10mRT), timed rising from the floor from lying (Gowers' manoeuvre).

Side effects

Side effects recorded include objective evaluations carried out during clinical appointments (i.e.: weight, height, blood pressure, whole spine bone density measurements, vertebral fractures), and adverse events reported by the families/boys and logged by the physician as mild, moderate or severe (i.e.: first presentation or aggravation of behavioural problems, insomnia, abdominal pains, gastroesophageal reflux, increased appetite, history of infections)

according to the instructions provided in the clinical forms, which were discussed and agreed at national consensus meetings organised at the inception of the network.

Table 6. The 17 items of the NorthStar Ambulatory assessment

NorthStar Ambulatory Assessment
Stand
Walk
Stand up from chair
Stand on right leg
Stand on left leg
Climb box step -right leg
Climb box step -left leg
Descend box step -right leg
Descend box step -left leg
Gets to sitting
Rise from the floor
Lift head
Stand on heels
Jump
Hop – right leg
Hop –left leg
Run (10 metres)

Statistical methods

I described the general patient characteristics for each GC regimen. As this is an observational study and some patients switched regimens throughout the course of their treatment, comparisons between regimens were analysed in three ways: 1. as per initial treatment, daily vs intermittent; 2. by majority of treatment, daily vs intermittent, as defined by the overall majority of time treated with one regimen (≥ 60% of time); 3. by removing switchers. Results for these three approaches were very similar for all analyses; therefore, we have presented final results for the most clinically relevant "majority of treatment" (daily or intermittent). Switchers were too heterogeneous to be grouped as one cohort of patients for the purposes of the analysis.

For the time to loss of ambulation (LOA) a Cox regression model was used to compare the effect of daily versus intermittent. An adjustment was made for random centre effects and hence the hazard ratio is presented along with a 95% CI, derived using robust standard errors. For the main longitudinal functional outcome measure NSAA score, a running line smoother (i.e. lowess smoother) [128] was used to provide a graphical representation of a locally weighted regression of a dependent variable (i.e. age) on a predictor variable (i.e. NSAA) . This revealed a definite change in the relationship after about age 7, which was in line with what observed clinically and reported before.[113, 129] A piecewise linear spline[128] with a single knot (changing point) at age 7 was used to allow for this effect: in other words we fitted a function defined by 2 sub-functions (one under boys age 7 and another function for boys above age 7. The spline, which is a smooth linear function,[128] was incorporated into a two level multilevel model with the random effect of patient nested within random centre. This allowed comparing the longitudinal effect of age between daily and intermittent, both

before and after age 7 years, by fitting two interaction terms for age and regimen. We fitted a similar model for 10mRT and the timing for the Gowers' manoeuvre; as this data was skewed, a log transformation was used. The longitudinal models were adjusted for the length of time on steroids prior to entry to the database. Additionally, we hypothesised that BMI may be related to outcome and therefore explored the models adjusting for BMI z-score. As BMI data was unavailable for some observations (13%) we used multiple imputation with the method of chained equation to generate five imputed data sets, so rather than a single value being imputed for every missing value, each missing value is imputed in a series of imputed data sets and these are combined together.[130] This was done assuming the data was missing at random, which means that the probability that a value is missing depends only on observed values and not on unobserved values.[130] Estimates obtained from the multiple imputations were pooled to obtain a single set of results.

We compared the NSAA total score in boys who started GC before age 5 versus after age 5 with a multilevel model adjusting for treatment. For respiratory and cardiac outcome measures a linear multilevel model was used to investigate the relationship of Forced Vital Capacity percentage predicted (FVC%) and Left Ventricular Shortening Fraction percentage (LVSF%) respectively, with age and the interaction with treatment regimen.

Side effect profiles reported by families and, where possible measured during the consultation, were compared as proportions by $\chi 2$ analysis. The BMI was calculated from height and weight. Height, weight and BMI centiles for gender and age were derived against the British 1990 Growth Reference and converted to z-scores (LMS Growth Calculator).[131] BMI z-scores were described as mean and 95% CI. At time of latest follow-up, differences between intermittent and

daily BMI, height and weight z-score means were compared by regression analysis adjusted for length of time on steroids. Single measurements at last follow-up were used for patients on treatment more than one year. BMI z-score means were compared for intermittent and daily at baseline using a two-sample t-test. Baseline included pre-GC single measurement to a maximum of three months into treatment. Hypertension was defined as a blood pressure >95th percentile.

For all tests, a p-value of ≤0.05 was considered statistically significant. The statistics package *Stata*[132] was used for the analysis.

Results

Longitudinal data on 360 patients were included in the analysis (figure 5, table5). Seventy-two percent of the patients registered in the database were recruited in the study. The mean age of starting GC was 6.4 years (range 3.4, 9.8) and mean duration of treatment and follow-up was 3.9 years (range 0.5, 8.5). Intermittent (n=154) and daily (n=136) were the most common regimens, used in 73% of boys on GC; additionally 70 of 72 switchers were treated with daily or intermittent prednisolone (table 7B). The general characteristics of intermittent, daily and switchers were similar in relation to age starting steroids, mean dose of GC, duration of treatment, duration of follow-up as NSCN registered patients (table 7A). The mean dose of GC was an overall group mean through the full period of treatment, which included dose adjustment in line with weight and tolerability. A total of 28 boys discontinued GC.

Table 7. General characteristics of patients

A.

	Age of diagnosis (years) mean, range	Age of starting GC (years) mean, range	Duration of treatment (years) mean, range	Dose of GC (mg/kg/day) mean, range	Duration of follow-up (years) mean, range
IP (n=154)	4·4 (0·3-9·4)	6·5 (4·2-9·6)	3·6 (0·5-8·5)	0·6 (0·3-0·8)	2.5 (0.2-5.4)
DP (n=136)	4·3 (0·3-9·4)	6·2 (4·2-9·8)	4·3 (0·5-7·5)	0·5 (0·3-0·8)	2.7 (0.3-7.8)
SW (n=72)	4·2 (0·2 -8·6)	6·3 (3·4-9·2)	4·1 (0·7-7·8)	0·6 (0·3-0·8)	3.2 (0.4-6.9)
DFZ (n=19)	5·5 (0·5-8·7)	7·0 (5·2-9·3)	4·4 (0·6-7·9)	0·6 (0·4-0·9)	2.8 (0.6-7.0)
AD (n=15)	4·4 (1·3-8·2)	6·3 (4·4-9·0)	5·0 (2·4-7·5)	0·6 (0·3-0·8)	2.0 (0.2-3.9)
Not on GCs (n=32)	3·3 (0·9-6·9)	----	----	----	----

B.

	IP	**DP**	**DFZ**	**AD**
Starting regimen	57	12	2	1
Majority of treatment	37	33	1	1
Age of switching regimen mean, ranges (years)	8·4 (5·6-12)	8·5 (7·3-9·3)	----	----

Table 7: General characteristics of patients

(A) Summary of patients' characteristics (n=428) with mean and ranges: age of diagnosis, age of starting steroids, duration of treatment, dose of steroids during the whole duration of treatment, and duration of follow-up since registration on the database. (B) Switchers (n=72)**:** In the analysis 70 switchers were included as per majority of treatment for intermittent and daily.

GC = Glucocorticoid corticosteroids; IP = intermittent prednisolone 10-dayson/10-days-off, DP =daily prednisolone, SW =switchers, DFZ =deflazacort; AD =prednisolone on alternate days.

Figure 6. Survival curve on loss of ambulation

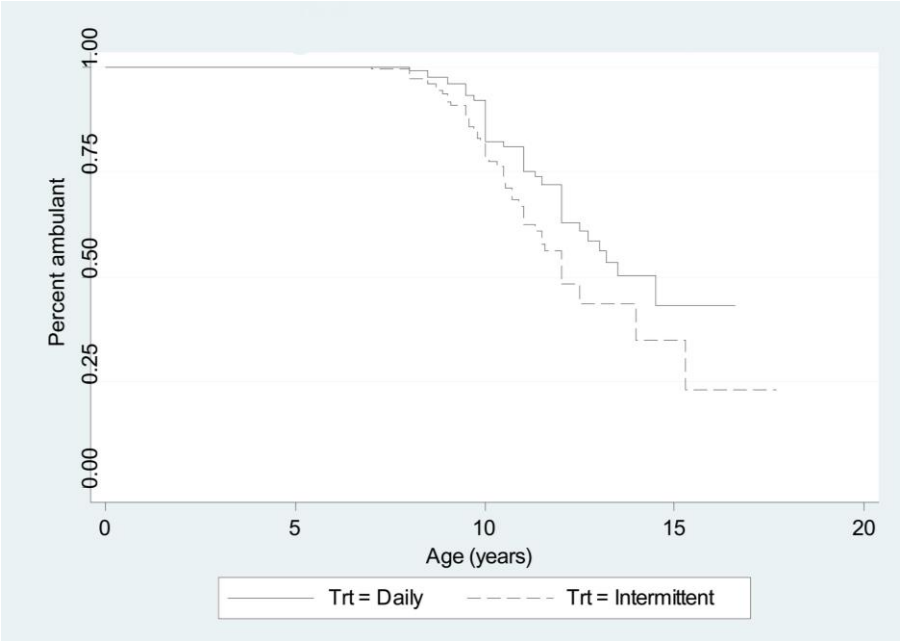


Figure 6: Kaplan-Meier analysis estimates for loss of ambulation and Cox regression

Loss of ambulation was reported in 48/176 intermittent prednisolone, and 36/165 in daily prednisolone. Median loss of ambulation intermittent =12 years, daily =14.5 years. Hazard ratio 1.57 (95% CI 0.87, 2.82) p=0.13.

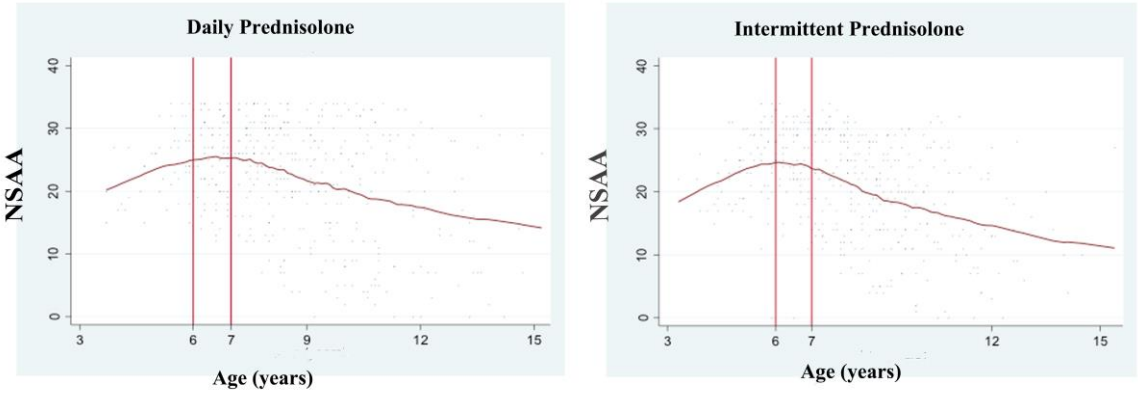
Outcome measures

Loss of ambulation (LOA) was reported in 51/184 intermittent and 39/168 daily (figure 6). Two boys were never ambulant and excluded from this part of the analysis; ambulation status was unknown for the remaining six. Median LOA was 12 years for intermittent and 14.5 years for daily. Hazard ratio for intermittent versus daily was 1.57 (95% CI 0.87, 2.82; p=0.13) and the mean age for LOA did not differ. The longitudinal analysis of NSAA total score showed a difference in the relationship with age between the two regimens after 7 years of age (figure 7). Daily showed the slowest decline, with the difference between the 2 regimens increasing by 1.58 units per year, 95% CI 1.04, 2.11; p<0.001. This result was consistent when adjusting additionally for BMI z-score. Adjusting the NSAA total score model for severe learning difficulties, we observed an overall difference in means (2.74 NSAA points, 95% CI 0.37, 5.10; p=0.02), such that patients with learning difficulties performed worse. There was little change in the relationship with age between intermittent and daily (coefficient 1.36, 95% CI 0.79, 1.94; p<0.001), compared with the model without learning difficulties. Comparison of the NSAA total score between patients who started GC over and under age 5 years, after adjusting for regimens, demonstrated an overall motor function improvement in favour of children who started early treatment (difference =3.04, 95% CI 0.15, 6.23; p=0.06). For the 10mRT outcome, there was evidence of a difference in the relationship over time between the two regimens after age 7 years, favouring daily (figure 8A). The difference increased by 6% per year on average, 95% CI 3%, 9%; p<0.003. A similar clinical observation was found for time rising from the floor (Gowers' manoeuvre) with a difference after age 7 of 6% per annum, 95% CI 0%, 12%; p=0.06 (figure 8B). After adjusting for BMI z-score the size of this effect decreased for both outcomes.

We also described the slope of decline for respiratory and cardiac function. Respiratory and cardiac outcomes were not different between intermittent versus daily regimens (Figure 9). The entire cohort (daily and intermittent together) was then analysed as one group. Within the age range of the cohort, mean values remained within normal limits. However, there was a significant progressive decline in the FVC% by 2.2% per annum ($p<0.001$) after age 10 years; and in the LVSF% by 1% per annum ($p<0.001$) after 12 years old.

Figure 7. North Star Ambulatory Assessment total score intermittent versus daily prednisolone

A **B**



C

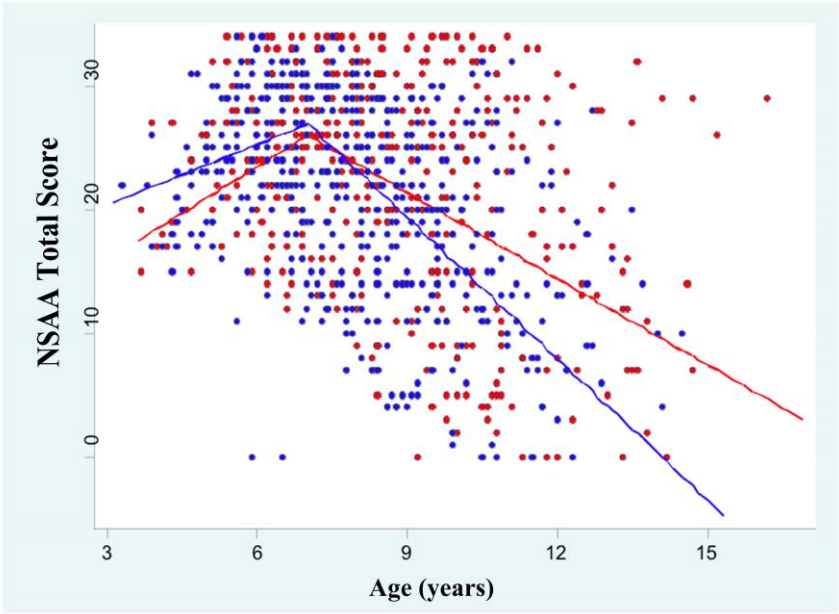


Figure 7: North Star Ambulatory Assessment total score intermittent (blue) versus daily (red) prednisolone

(A) NSAA total score running line smoother for daily and (B) intermittent prednisolone. NSAA decline began at 7 years in the daily group and at 6 years in the intermittent group. (C) A fitted multilevel model and interaction for the NSAA total score was calculated comparing intermittent and daily. There were 862 episodes. There was a definite change in the relationship after 7 years of age:

< 7 years interaction coefficient -0.81 (95% CI 0.42, 2.04), p=0.2; ≥7 years interaction coefficient -1.58 (95% CI 1.04, 2.11) p<0.001. Intermittent deteriorates faster than daily after 7 years of age. For each additional year, the difference in NSAA total score between the 2 regimens increases by 1.58 points.

Figure 8. 10 metres run time and time rising from the floor: intermittent versus daily prednisolone

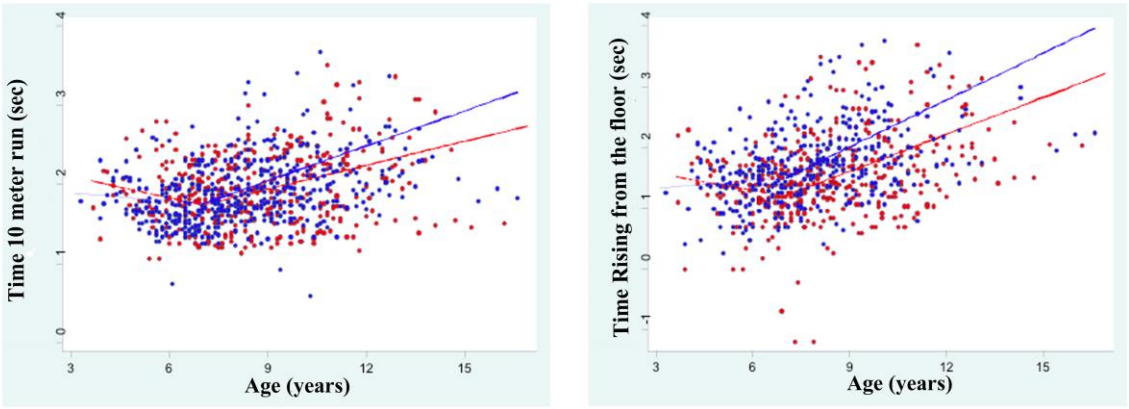


Figure 8: 10 metres run time (A) and time rising from the floor (B) intermittent versus daily prednisolone (log secs)
Daily prednisolone (in red) shows a slower progression in both timed tests

Figure 9. Left Ventricular Shortening Fraction percentage and Forced Vital Capacity percentage: intermittent versus daily prednisolone

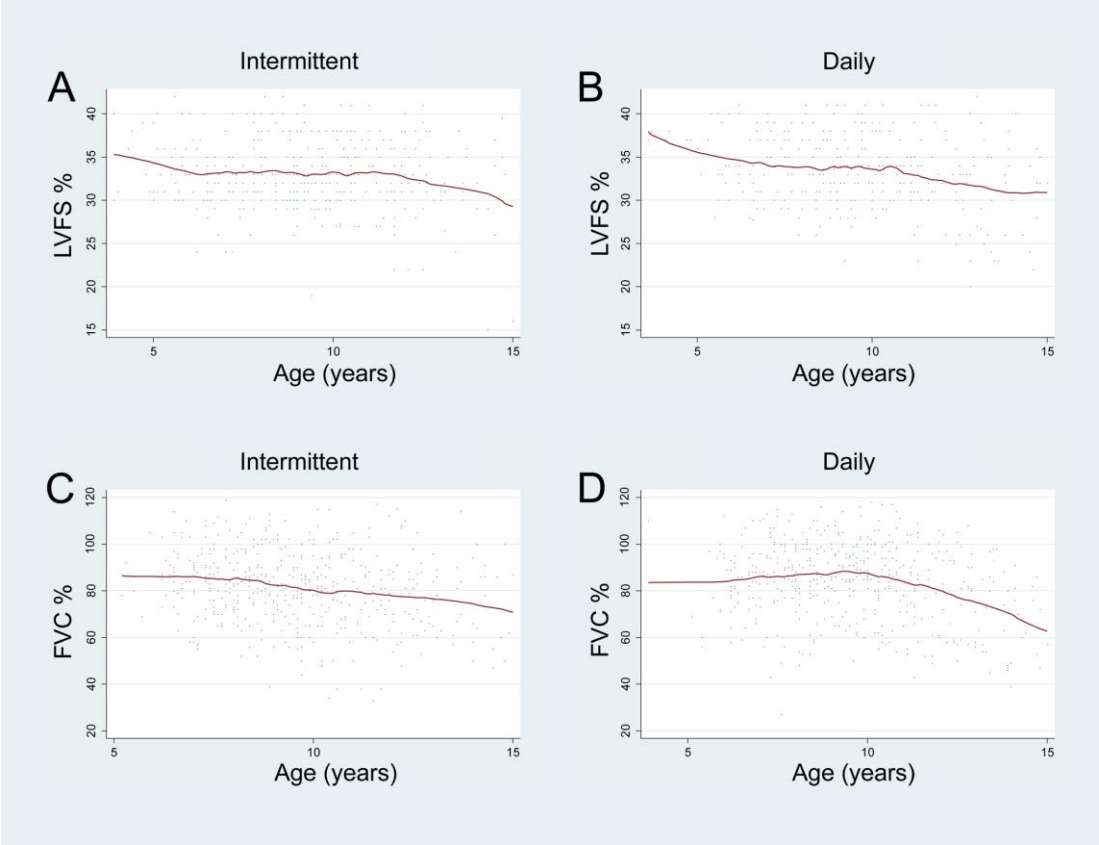


Figure 9: Left Ventricular Shortening Fraction percentage (LVSF %) and Forced Vital Capacity percentage (FVC %): intermittent versus daily prednisolone
(A) Left Ventricular Shortening Fraction percentage running line smoother for intermittent and (B) daily prednisolone. There were 683 episodes. There was no evidence for a difference in slopes between regimens (p=0.11). The relative reduction was 1% per annum (p< 0.001) for both groups. (C) Forced Vital Capacity percentage running line smoother for intermittent and (D) daily prednisolone. There were 964 episodes. There was no evidence for a difference in slopes between regimens (p=0.16). The relative reduction was 2.2% per annum (p< 0.001) for both groups.

Side effects

Side effects were summarised for intermittent and daily prednisolone, assigning switchers by "majority of treatment" (table 8).

Moderate and severe side effects were more frequently observed in daily GC. Statistically significant differences were found in daily/intermittent respectively: Cushingoid features 33%/15%, hyperactivity 23%/15%, gastro-intestinal symptoms 14%/6%, and hypertension 22%/5%. Severe side effects alone were not significantly higher in the daily group. Baseline BMI did not differ between intermittent and daily (figure 10 A), both groups gaining excessive weight (figure 10 D), however the mean height z-score, adjusted for length of time on steroids was significantly lower in daily, with a mean difference of 1.09 (95% CI 0.78, 1.40; $p<0.001$) (figure 10 C). The overall effect at latest follow-up was a significant increase from baseline in BMI in both regimens, but daily more severely affected than intermittent with a mean difference 0.43 (95% CI 0.11, 0.74); $p<0.01$ (figure 10 B). Bone health was compromised in both intermittent and daily GC (table 9): BMD z-scores ≤-2.5 on DEXA scan were observed in 5 % in intermittent 8% in daily. Vertebral fractures were defined by the NSCN as vertebral wedging on lateral spine radiography.[57]

Table 8. Moderate to severe side effect breakdown

SIDE EFFECTS	Intermittent n (%)	Daily n (%)	χ^2 p-value
Temper tantrums	54 (28%)	67 (40%)	0·02*
Mood swings	56 (29%)	64 (38%)	0·08
Aggressiveness	41 (21%)	49 (29%)	0·09
Hyperactivity	29 (15%)	39 (23%)	0·05*
Emotional liability	23 (12%)	32 (19%)	0·06
Insomnia	8 (4%)	19 (11%)	0·01*
Cushingoid features	28 (15%)	56 (33%)	< 0.01*
GI symptoms	12 (6%)	23 (14%)	0·01*
Increased appetite	73 (38%)	78 (46%)	0.1
Hypertension	10 (5%)	38 (22%)	< 0·01*
Vertebral fractures	8 (4%)	14 (8%)	0·1
Long bone fractures	13 (7%)	9 (5%)	0·5
§BMD z-score ≤ -2.5	9 (5%)	14 (8%)	0·1
Cataracts	2 (1%)	4 (2%)	0·3
Hirsutism	19 (10%)	24 (14%)	0·2
Easy bruising	5 (3%)	7 (4%)	0·4

Table 8: Moderate to severe side effect breakdown, χ^2 analysis (intermittent prednisolone n=191; daily prednisolone n=169).

§BMD z score = lumbar spine

Figure 10. BMI, height and weight: intermittent versus daily prednisolone

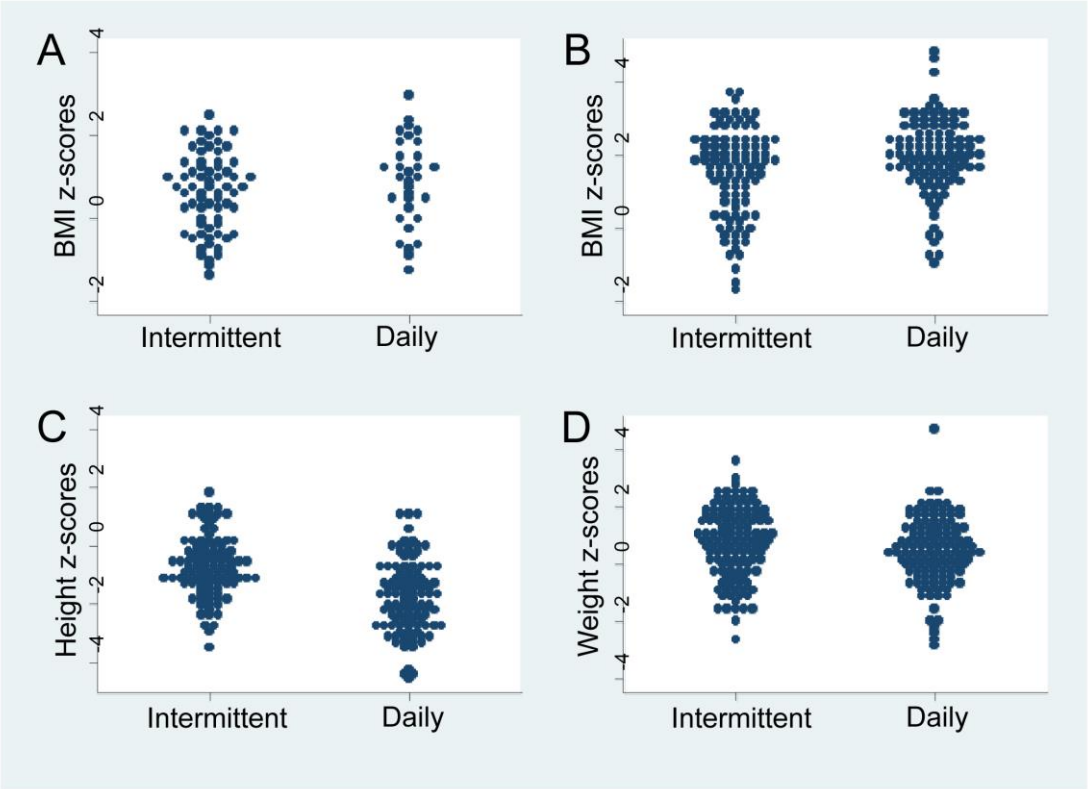


Figure 10: BMI, height and weight: intermittent versus daily prednisolone:
(A) BMI z-score (mean, ± 95% CI) at baseline: there was no significant group difference between BMI. Intermittent (n=67) baseline mean z-score 0.62 (95% CI 0.39, 0.85), daily (n=32) baseline mean z-score 0.88 (95% CI 0.50, 1.27), p=0.2. (B) At maximum period of treatment§ there was a group difference in BMI favouring intermittent (n=99) versus daily (n=102): Intermittent BMI mean z-score 1.51 (95% CI 1.27, 1.75), daily 1.99 (95% CI 1.79, 2.19), p=0.002. (C) Height restriction was significant in daily (n=104) but there was no group loss of height for intermittent (n=101): Intermittent height mean z-score -0.70 (95% CI -0.90, -0.49), daily -1.77 (95% CI -2.00, -1.53), p<0.001. (D) Weight z-score (mean, ± 95% CI) at maximum period of treatment: intermittent (n=123) weight mean z-score 0.79 (95% CI 0.57, 1.01), daily (n=122) 0.50 (95% CI 0.29, 0.71) p=0·06.
§Maximum period of treatment= single measurement at last follow-up of patients at least one year into treatment.

Discussion

Here I report the largest prospectively collected multicentre longitudinal clinical data of ambulant DMD boys treated with GC according to internationally agreed standards of care. In particular, I describe the long-term efficacy and tolerability of intermittent versus daily prednisolone in 360 UK-treated DMD boys, on treatment for a mean of 4 years. In this study I included 191 boys on intermittent and 169 boys on daily prednisolone. Switchers (n=70) did not affect results, and were analysed by "majority of treatment" regimen.

Survival analysis of median LOA favoured daily treatment (14 years) with two years advantage compared to intermittent (12 years). However, the hazard ratio was not statistically significant because the mean ages were not different. DMD boys lose ambulation by the end of the first decade if untreated,[45, 133] however daily therapy has been shown to prolong ambulation beyond 13 years of age.[42, 45] Additionally, the implementation of the international standards of care endorsed by the NSCN and NICE, through addressing the multi-system requirements of DMD, may also significantly contribute to prolongation of ambulation.

I demonstrated that the two regimens equally gain function until 6 years but after age 7, boys on intermittent GC decline more rapidly with an incremental difference (NSAA score 1.58 per annum). There was also a faster decline in the 10mRT and Gowers' manoeuvre: 5% and 6 % respectively per annum. Boys with severe learning difficulties performed worse on motor assessments, but this was not affected by steroid regimen. Our data indicate a clinically significant benefit in starting GC early, fuelling the on-going debate on how early treatment should be started.[134] A recent long-term case series of four DMD boys treated between age 2-4 reported prolonging ambulation beyond 16 years.[135] In my study, boys

who began treatment before 5 years of age showed a trend towards slower functional decline.

Despite the relatively young age of the cohort, in the second decade respiratory and cardiac outcomes showed a decline of LVSF% by 1% and FVC% by 2.2% per annum, irrespective of treatment regimen.

A larger proportion of patients on daily GC reported moderate to severe side effects. Cushingoid features (33% vs 15%), behavioural problems (40% vs 29%) and hypertension (22% vs 9%) were significantly higher in daily GC treatment. We highlighted a significantly increased BMI in daily (mean z-score 1.99) versus intermittent (mean z-score 1.5), and severe height restriction in daily (mean z-score -1.77) compared to intermittent treatment (mean z-score -0.70).

Long-term use of GC is a recognised and well-described risk factor for decreased bone mineral density and increased incidence of vertebral fracture in DMD.[54, 55, 57, 136] In our studied population, symptomatic vertebral fractures were seen in 8% on daily and 4% on intermittent. A prevalence of up to 32% has previously been reported in smaller series of chronically GC-treated DMD boys.[137] Asymptomatic vertebral fractures are possibly less likely to be ascertained by the NSCN database. NSCN DEXA scan data (table 4) suggest that applying the standard of care reduces osteopenia, affecting only 8 % of boys on daily prednisolone.

Nascent and evolving guidelines on bone protection in DMD recommend: normalisation of serum 25-hydroxyvitamin-D3 with oral supplements at diagnosis, close surveillance while on GC, annual DEXA and intravenous bisphosphonates for symptomatic vertebral fractures.[57] Furthermore, osteopenia/Z-scores ≤-2.5 require a lateral spine radiograph looking for vertebral deformity, which may require bisphosphonate treatment.[57] The NSCN aims to incorporate these recommendations for the future.

A number of prospective studies have been published on GC treatment in DMD, ranging from cohort studies to randomised-double-blinded clinical trials (see table 3). Only two studies compared daily with intermittent GC in the short-term.[113, 114] A recent randomised-blinded clinical trial compared high dose weekend prednisone (10 mg/kg/week) vs standard daily dose (0.75 mg/kg/d) over 12 months in 64 DMD boys.[114] The study provided evidence that high dose weekend prednisone is equally effective in preserving muscle function for some of the outcome measures, but with increased linear growth and lower BMI.[114]
A recent longitudinal multicentre cohort study reported NSAA changes over 12 months on 106 ambulant DMD boys. A clear slope of change was observed at the age of 7, also confirmed by our findings; above age 7 (n=71), a slower rate of decline was reported in boys on continuous GC. No randomised controlled study has previously reported data on LOA in DMD boys treated with an intermittent regimen.[113]
Our study has several limitations: incomplete and partially missing data, adjusted for in the statistical analysis; outcome measures and side effect profile were analysed collectively and not as single patient trajectories; side effects were assessed in clinic and included parental reports; there was no validated quality of life information; there were no validated measures for learning difficulties and behavioural problems; and analysis did not adjust for severity of phenotype at enrolment. Furthermore, it is difficult to account for the effect of GC treated boys whose parents did not consent, or for incomplete data. Finally, we could not compare outcomes with an untreated cohort, as almost invariably families would agree on starting GC. Following this study, a more rigorous definition of side effects will be introduced in the NSCN, particularly concerning behavioural problems, using validated psychometric evaluations. Nevertheless, for the first

time our analysis provides long-term outcome data from diagnosis to LOA in a large sample representative of the ambulant DMD population in the UK. These data offers evidence for a functional benefit of intermittent prednisolone, possibly more so if initiated early, delaying median LOA to 12 years. This contrasts to the very modest effect of intermittent GC given to older boys.[42] The intermittent regimen was overall better tolerated with fewer adverse effects. Although multiple randomised controlled clinical trials (RCTs) remain the gold standard in determining therapeutic safety and efficacy, there is an increasing recognition of the importance of observational studies.[138-141] A robust well-designed and properly analysed secure database containing prospectively and systematically acquired data can be a valuable tool for guiding evidence based decisions in relation to treatment and in designing future controlled trials.[142] Our study provides a framework for consultation when starting treatment, however does not offer a definite answer on which GC regimen should be used, and a long-term RCT of intermittent vs continuous GC is required. The NSCN is collaborating in an international clinical trial of different steroid regimes funded by the National Institutes of Health (FOR-DMD: Finding the Optimum Regimen of Corticosteroids for DMD), which will compare daily prednisone/deflazacort regimens versus intermittent in a young steroid-naïve population in a randomised controlled trial.[143]

A future challenge will be to assess the efficacy and safety profile of continuing GC administration in the non-ambulant population. With the implementation of multidisciplinary interventions, in particular respiratory management, life expectancy has shifted to the third/fourth decades compared with the second/third decades for steroid-naïve patients.[29] GC in DMD is likely to further prolong life. Longitudinal analysis is required to evaluate survival and quality of

life in DMD, and determine the optimal treatment protocol in continuing GC treatment beyond LOA.

In conclusion, our study comparing benefits and tolerability of the most widely used GC regimens in the UK addresses one of many facets of a multi-system disorder, which is evolving. Further prospective collection of clinical data with a robust and refined tool can significantly facilitate monitoring and improving the standards of care for this common genetic disease.

CHAPTER 3: VARIABILITY OF PHENOTYPE IN DMD: IMPLICATIONS FOR CLINICAL CARE AND CLINICAL TRIAL DESIGN

The work contained in this chapter has given rise to the following publication: **Ricotti V**, Ridout D, Scott E, Mayhew A, Main M, Manzur AY, Muntoni F, on behalf of NorthStar Clinical Network . *The Northstar ambulatory assessment in Duchenne muscular dystrophy: considerations for the design of clinical trials* J Neurol Neurosurg Psychiatry. 2015 Mar 2. [Epub ahead of print]. PMID: 25733532

My contribution to this work consisted of the following: I oversaw the design and conduct of the study, and analysis. I led the analysis of the data in collaboration with the statistical department at UCL, ICH. I wrote the first draft of the manuscript, and I contributed to the revision of the manuscript.

Experimental therapies in DMD

In recent years a number of experimental therapeutic approaches have been developed. These can be divided in three major groups:

1) Those aiming at restoring the absent dystrophin protein in muscles, and mostly targeting specific mutations of the *dystrophin* gene. These therapeutic approaches include:

- exon skipping mediated by antisense oligomer nucleotides,[78-81]
- read-through of intragenic stop codon mutations by small molecules,[144, 145]

2) Other non-mutation dependent therapeutic approaches include:

- ibedenone, a potent antioxidant, which proved to be effective in stabilising pulmonary function in non-ambulant subjects in a phase 3 trial;[146]
- tadalafil, which through the inhibition of the nitric oxide –cyclic guanosine 39,59-monophosphate pathway promotes muscle blood flow, and proved to alleviate functional muscle ischemia in a small cohort of DMD boys.[147] Currently in a phase III clinical trial;
- up-regulation of utrophin by a novel small molecule; utrophin is a close evolutionary relative of dystrophin. Up-regulation of this protein with SMT C1100, an orally available small molecule in animal models proved to compensate for the lack of dystrophin in skeletal muscle in the *mdx* mouse.[148] Currently a phase Ib clinical trial is on-going.

3) And finally viral-mediated gene therapy, which can be used as replacement therapy, for example using a mini-dystrophin[149-151] or to correct specific mutations, for example through U7- or U1-mediated exon skipping.[152-154]

Mutation specific therapeutic approaches

Approximately 70% of dystrophin mutations are deletions that affect the normal splicing of the dystrophin RNA transcript into mRNA. As a result the normal dystrophin mRNA open reading frame is disrupted leading to a prematurely truncated dystrophin protein. The abolished production of a functional protein is the underlying cause of the disease.

Mutations in the *DMD* gene are also found in Becker Muscular Dystrophy (BMD), however characterised by a much milder phenotype when compared to DMD.[27, 155] This milder phenotype occurs because the BMD mutations generally affect the centre of the gene and maintain the open reading frame, which in turn allows the production of a shorter but functional protein product.[156] Clinical observations of BMD patients, who remained ambulant until old age or did not develop muscle weakness at all despite expressing 30-60% of dystrophin on muscle biopsy, fuelled the idea that restoring at least a partially functional protein can ameliorate the disease course of DMD. An approach to restore normal mRNA reading frame is by exon skipping, which can be induced by antisense oligonucleotides (AONs), synthetized fragments of nucleic acids, which modulate RNA splicing in the pre-mRNA at the proximity of the mutation, resulting in a shorted mRNA, which however carries a normal open reading frame and can produce a partially functional BMD-like protein (figure 11). Two types of chemistries have advanced into clinical trials: phosphorodiamidate morpholino oligomers and 2'-O-methyl RNA with a phosphorothioate backbone; a review of the chemical properties and differences has been published by Arechavala-Gomeza and colleagues.[157] The recent drug developments have targeted exon 51 of the DMD gene, as this could be applied to approximately 14% of the mutations resulting in DMD (see table 9).[20] Proof of concept studies showed dystrophin restoration by local and systemic administration.[76-79] Therapeutic efficacy and safety profiles have been established and reported in recently published phase II studies.[80, 81] AONs targeting other exons (i.e. 44, 45, 53,) are also currently being developed, and both chemistries are now in phase I-II clinical trials.

An alternative approach includes delivering an antisense sequence linked to a U1 or U7 snRNA gene packaged into a recombinant adeno-associated virus vector, which showed promising results in animal models,[154] and a clinical trial targeting exon 53 is currently being planned.

Figure 11. Schematic representation of the principal of exon skipping

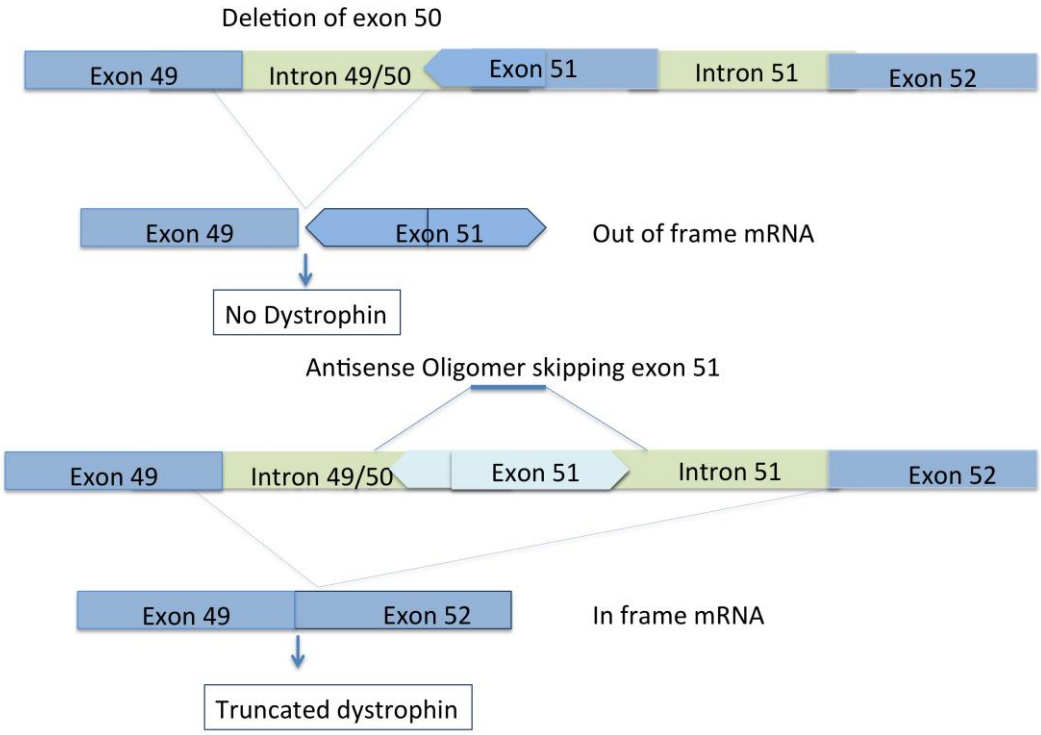


Figure 11: Schematic representation of the principal of exon skipping
In the top image a deletion of exon 50 leads to the disruption of the *m*RNA reading frame. No protein is produced.
In the bottom figure, an antisense oligomer targeting exon 51 in the pre-*m*RNA and preventing its inclusion into the *m*RNA restores the open reading frame leading to the production of a truncated but functional protein.

Table 9. Applicability of single exon skipping

Exon	Applicability for deletion	Applicability for all mutations
51	20.5%	14.0%
45	13.1%	9.0%
53	11.8%	8.1%
44	11.1%	7.6%
50	5.6%	3.8%
43	4.5%	3.1%
8	2.9%	2.0%
55	2.5%	1.7%
52	1.3%	0.9%
11	1.3%	0.9%

Table 9: Applicability of single exon skipping
Deletions that can be corrected by skipping of either of the flanking exons (i.e: deletion exon 52) were not counted twice. Source[20]

Approximately 13% of subjects with DMD carry an intragenic nonsense mutation of the *dystrophin* gene.[20] A nonsense mutation results in a premature stop codon within the protein-coding region of the corresponding mRNA and causes premature termination of translation, which therefore results in on of a non-functional protein product. An alternative strategy to exon skipping, which aims at restoring dystrophin in patients with eligible mutations, is ribosomal read-through mutation suppression of the premature stop codon. As amino acid inclusion at the nonsense mutation-carrying codon is a chance process many of the dystrophin proteins thereby created are expected to carry missense mutations. A proof-of-concept phase IIa study demonstrated that ataluren/PTC 124 produced dystrophin in patients with nonsense mutations;[158] a subsequent Phase IIb registration-directed study showed patients receiving treatment at a lower dose experienced significantly slower progression when compared to placebo.[82] Currently the compound is being evaluated in a phase III clinical trial.

Gene replacement therapy

Replacement of the defective dystrophin gene offers another promising approach, which can potentially be life-prolonging. This strategy is currently in pre-clinical phase of development and planned to go forward to the clinic in 2017.

The design of clinical trials in Duchenne muscular dystrophy

The development of therapies for DMD and the rapid explosion of clinical trials, especially in the past 6 years, have triggered the requirement for some important considerations about the design of clinical trials.

The overall natural history of DMD has significantly changed in the recent years. With the introduction of glucocorticoid (GC) therapy and systematic implementation of the standards of care, age at loss of ambulation (LOA) and life expectancy is gradually shifting to a later age, requiring continuous monitoring of the evolving clinical course DMD.[43, 159-161] In addition, mutation-specific therapeutic approaches, as discussed above, have generated the need for understanding the natural progression of the targeted genotype subgroups, especially if differences from the overall DMD population exist. It is recognised, for example, that some patients with mutations skippable by exons 44 or 45 may present an intermediate phenotype, due to an elevated number of revertant fibres and residual dystrophin expression;[162, 163] however, the influence of the different genotypes on disease course appears to be modest according to a recent study.[129] Furthermore, the course of DMD can also be influenced by the age at starting GC and GC regimen[42, 35] The standards of care suggest initiating GC in the plateau phase;[32 33] the additional benefit of starting therapy at an younger age has been reported on a small case-series of children[135] which invites further exploration.

Clinical trials design of rare progressive disorders, such as DMD, poses various challenges: 1) the number of patients who can be recruited into studies is often limited. In case of exon skipping recruitment is mutation-specific, making it problematic to run large and well-powered studies; 2) cohorts may be too small to allow for a placebo arm, and small cohorts may not be representative of the natural variability even within a genotype, thereby leading to skewed results; 3) validated outcome measures may only capture a snapshot of the progression of the disorder and not be applicable to different stages of the condition, further limiting the number of patients recruitable into studies (e.g. the 6-minute walk

distance test,[164] a validated primary outcome measure in a number of clinical trials, can only be performed in ambulant individuals of at least 4 to 5 years); 4) most of the future genetic therapy approaches will target small cohorts due to high costs of experimental therapies and the challenges of running large multinational studies and will also need long-term follow-up to allow ascertainment of potential side effects, thereby emphasizing the need for precise subgroup characterization.

Large natural history studies, if systematically conducted, can meaningfully inform the design of clinical trials, by offering an up-to-date description of the progression of the condition according to concurrent standards of care, and a valid platform of natural history data to guide both inclusion criteria and outcome measure selection.

In this study, which focused on the NorthStar Ambulatory Assessment (NSAA) as a measure of motor function, our objectives were the following: 1) to assess the NSAA evolution in ambulant DMD boys in the UK treated according to the agreed standards of care; 2) to describe the rate of progression of a subgroup of young DMD boys in the UK treated with GC below five years of age; 3) to describe the NSAA rate of decline in DMD stratified for *DMD* genetic mutations including individuals in the UK and in the Italian neuromuscular clinical network .

A study on the NorthStar ambulatory assessment in Duchenne muscular dystrophy: considerations for the design of clinical trials.

Methods

Through the UK NorthStar Network and database, which encompasses 17 neuromuscular centres, clinical data from 2004-2012 on 513 ambulant DMD boys between 3 and 16 years of age (mean age 9.5 years) were included in the analysis. The database systematically collects clinical information on DMD boys in UK. The diagnosis is confirmed in most cases by DNA diagnostic technique covering all DMD gene exons, and/or a muscle biopsy. Mutations were classified according to the Leiden Muscular Dystrophy database.[123] All patients included in the analysis were treated according to the standards of care,[32, 33] comprising therapy with GC administered ether as daily prednisolone/deflazacort or intermittently (ie: prednisolone 10 days on:10 days off or alternate days[35, 42]). For the analysis of the young DMD patient, I addressed the evolution in UK boys from 3 years onwards, comparing those who started GC before and after 5 years of age, as this would be a relevant population for future clinical trials.

NorthStar Ambulatory Assessment

The NSAA is a validated one-dimensional functional scale for ambulant DMD boys.[124, 125] The scale is suitable for multicentric studies,[124-126] and is widely used internationally, both in clinical setting and as secondary outcome measure in clinical trials. Both traditional and modern psychometric (Rasch) analysis has confirmed this to be a robust scale.[127] Recently an updated linearized version of the scale has been developed following in-depth Rasch analysis, to improve the

interpretation and capturing of clinical meaningful changes across the breadth of the scale.[165]

Statistical methods for the UK dataset

I used frequencies and percentages to describe the number of boys with each mutation. I described the median and other percentiles for age at LOA, along with 95% CI using the Kaplan-Meier method.

Previous work showed a difference in the relationship between motor function and age, up to 7 years of age compared with later stages in childhood[35, 43, 113, 129, 160, 161, 166]; therefore we decided to look at these 2 time periods separately for the purposes of this study.

NSAA score was our primary outcome, additionally I converted the raw NSAA total scores into linearized scores. Using Rasch methodology recently reported[165], the conversion was performed via a logit transformation, and this formed a linearized score on a 0-100 scale. I used this linearized transformation of the NSAA scale in all subsequent analyses and present the results in terms of this linearization. The benefit of this approach is that the change score means the same across the breadth of the scale. Furthermore analyses were repeated for the raw NSAA score scale and results are presented in brackets, as raw scores are perhaps more readily understood by clinicians. The transformed score was calculated using total NSAA score minus the score for lifts head (as this item poorly fits the construct of ambulation). With the number of items reduced from 34 to 32, the raw scores were converted to 100 points on the new scale as reported.[165] For some children the lifts head component of the NSAA scale was missing, therefore it was not possible to convert their total NSAA score to the

linearized score. I performed a sensitivity analysis considering several possible values for the missing lifts head score and derived the corresponding linearized scale. I found the results from these analyses were robust and there was no impact on subsequent findings. We fitted a separate multilevel model for each time period (data over age 7 years and data under 7 years), with a random effect for patient nested within random centre. I modelled the relationship between NSAA and age and adjusted for age at start of GC, and treatment regimen. In addition, by including interaction terms in our models we explored whether the rate of deterioration after age 7 or improvement in motor function up to age 7 varied according to the type of mutation: duplication, deletion or point mutation. For the early time period we explored whether there was a difference in improvement in NSAA between those boys who started treatment early (before age 5) and those who started later (between 5-6.5 years), by fitting an interaction term. Simple comparisons for items in the NSAA scale were made between early and late starters using t tests. Similarly, BMI z scores[131] were compared between early and late starters.

Fractional polynomials were used to check for any non-linearity in the relationship between independent factors and NSAA outcome.

Populations with skippable deletions - UK and Italian dataset

Genetic information was available for 442 of 513 UK boys (figure 12). Mimicking the setting of phase II and phase III clinical trials for the sub-analysis of the skippable genotypes, we included:

1) All DMD boys >5 years of age; 2) with a transformed NSAA total score of 52/100 or above at time 0/baseline (corresponding to ~ 230 metres 6MWD[159]; 3)

and on stable GC for a minimum of three months at baseline; 4) boys with at least 24 months longitudinal data.

This resulted in a reduced dataset of 223 DMD boys from UK, which was combined with 172 DMD boys followed-up by the neuromuscular Italian clinical network coordinated by the Department of Paediatric Neurology, Catholic University, Rome. Of the Italian DMD boys, 74 had specific skippable deletions: 20 with deletions exons 44/46 skippable; 1 exon 44 skippable; 17 exon 51 skippable, 1 exons 51 or 53 skippable; 25 exon 53 skippable; 8 exon 50 skippable; 2 exon 52 skippable.

Figure 12 . Genotype breakdown of individuals registered in the UK NorthStar database.

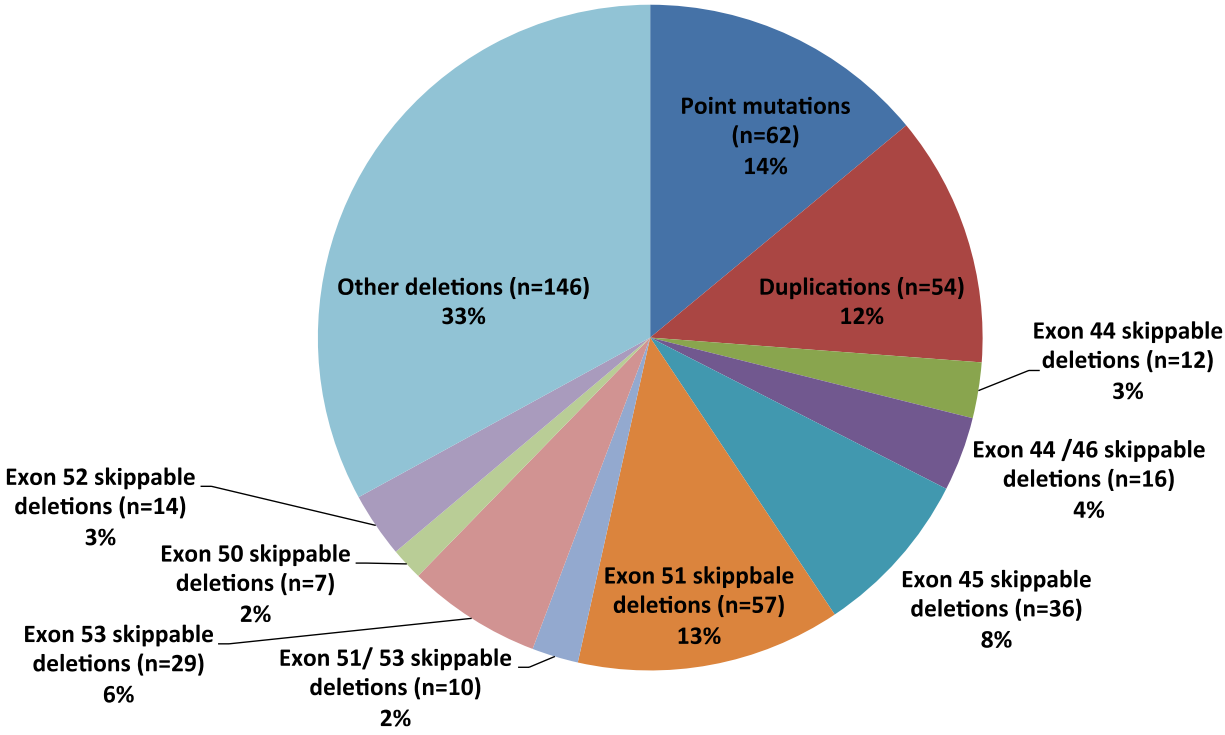


Figure 12: Genotype breakdown of individuals registered in the UK NorthStar database. Genotype information was available for 442 UK DMD boys.

Statistical methods for skippable populations

I compared the change in linearized NSAA over time between skippable exons for the UK and Italian data combined. For the Italian patients data was available at fixed time points, at 12 months and 24 months follow-up. We created a similar format of data from the UK NorthStar database using fixed data ± 1 month and combined the two datasets together (n=223+172=395 DMD). Since a small minority of DMD boys can benefit from skipping more than one exon, we fitted a series of multilevel models, each including two interaction terms to compare the rate of decline in NSAA between those boys with and without a particular exon skip. The first interaction term compared the decline over 12 months and the second the decline over 24 months. All models included a random effect for patient nested within country; we adjusted for age at follow-up and treatment regimen.

All analyses were conducted in *Stata* and for all tests a p-value of $< 0{\cdot}05$ was considered statistically significant.[132]

Results

UK NorthStar dataset

Age at loss of ambulation

In the UK NorthStar database, ambulation was lost in 137/513 boys between 9.5 and 16 years, including all GC treatment regimens. The median LOA was 13 years (95% CI: 12.1, 13.5). We also calculated the 10th centile: 9.5 years (95% CI: 9.1, 9.9); the 25th centile: 10.9 years (95% CI: 10.1-11.1); and the 75th centile: 16 years (95 %CI: 15 -na). (Figure 13, table 11)

Figure 13. Survival curve for loss of ambulation

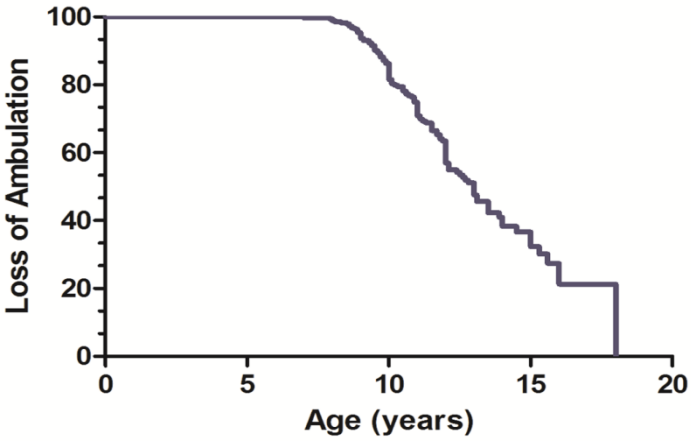


Table 10. Centiles for loss of ambulation

Centiles LOA	Age (years)	SE	95% CI
10th Centile	9.5	0.1	9.1-9.9
25th Centile	10.9	0.2	10.1-11.1
50th Centile	13	0.3	12.1 – 13.5
75th Centile	16	0.7	15-

NorthStar Ambulatory Assessment slope of decline in boys > 7 years of age

It was previously reported that DMD patients gain motor function up to age 7, after which they start to decline.[35, 43, 113, 160, 164, 166] In our cohort, the overall slope coefficient was -7.8 (95% CI -8.8, -6.9) [-3.7, 95% CI -4.1, -3.3], meaning that our DMD population on average lost 8 linearized NSAA units/year, after age 7 (figure 14). The estimated linearized NSAA score at age 7 was 73.4 (95% CI 70.3, 76.5) [27.4, 95% CI 26.1, 28.6] ($p<0.01$). When compared to the whole DMD population, a positive interaction coefficient 0.7 (95%CI: -1.9, 3.3) [raw NSAA:0.5 (95%CI -0.7, 1.7)] suggested a possible trend for a slower decline in boys with duplications ($p=0.5$), and a trend towards a more rapid decline in boys with point mutations (interaction coefficient= -1.5; 95% CI -4.1, 1.1; $p=0.2$) [raw NSAA:-0.3 95%CI -1.5, 1.0]; however neither of these differences was significant. In order to determine which NSAA score may best predict LOA within 24 and 12 months, assuming mean LOA of 13 years, we estimated the mean linearized NSAA score from our model, which in our population was 41.9 units, (95%CI 38.5, 45.3) [12.6, 95%CI 11.2, 14.1] 2 years prior to LOA, and 34.0 units (95% CI 30.1, 38.0) [8.9, 95% CI 7.2, 10.7] 1 year prior to LOA.

Figure 14. Linearized NSAA total score in DMD boys > 7 years of age

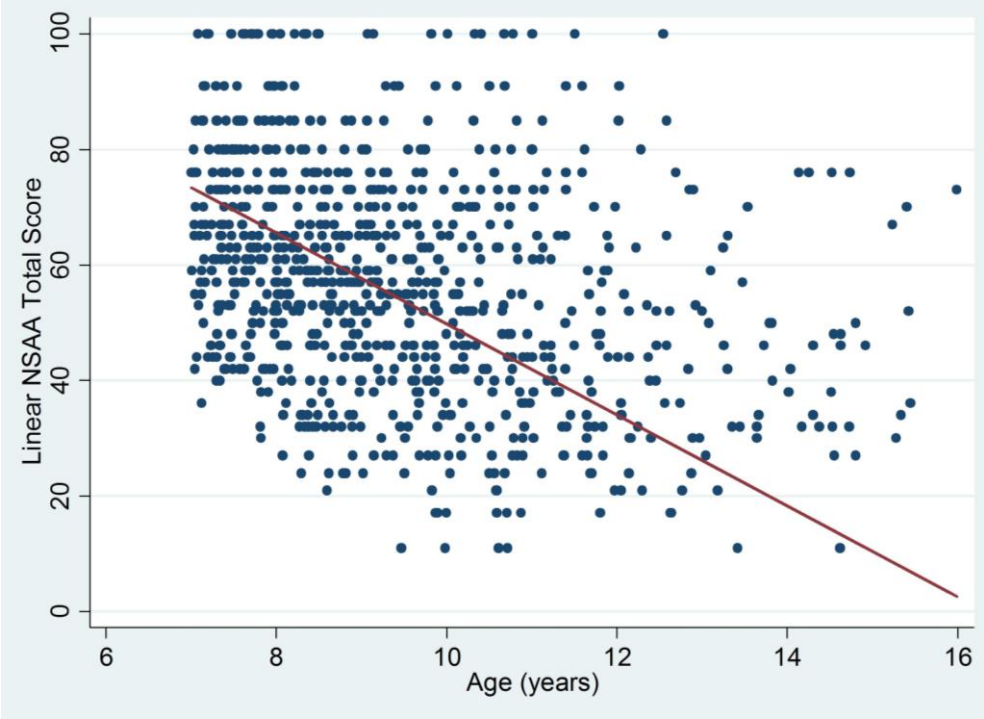


Figure 14. Linearized NorthStar Ambulatory Assessment slope of decline in boys > 7 years of age

With an average linearized NSAA score of 73.4 (95% CI 70.3, 76.5) at age 7 [raw NSAA: 27.4, 95% CI 26.1, 28.6] the overall slope coefficient was -7.8 (95% CI -8.8, -6.9) [raw NSAA: -3.7, 95% CI -4.1, -3.3], meaning that our DMD population on average lost 8 linearized NSAA units for each year, after age 7 ($p<0.01$). At 8 years of age the mean linearized NSAA was 65.5 (95% CI 62.7, 68.4) [23.7, 95% CI 22.6, 24.8], while at 10 years of age it was estimated to be about 49.7 units (95% CI 46.8, 52.8) [16.3, 95% CI 15.1, 17.5]. The mean linearized NSAA score was 42 units 34 units 24 and 12 months before losing ambulation [equivalent to 13 and 9 raw scores respectively].

NorthStar ambulatory assessment in young DMD boys

Prior to age 7, young DMD improve their motor function, with an overall gain of 4.0 linearized units/year (95%CI: 2.2, 5.8) (p<0.001) [1.4, 95%CI 0.6, 2.1], adjusting for treatment regimen. As part of this analysis we explored the impact that starting GC before age 5 may have on DMD as measured by the NSAA. When comparing 78 DMD who started daily or intermittent GC early, before age 5 (mean age at start =4.5 years) with 163 boys who started GC between ages 5 and 6.5 (mean age at start =5.7 years) the coefficient of interaction was -2.7, (95%CI, -6.8, 1.3, p=0.2), favouring early starters by almost 3 linearized units a year (figure 15) [Raw NSAA:-1.3, 95%CI -3.0, 0.3, p = 0.1]. By age 7, the mean total NSAA was different between the two groups (p<0.01): 73.8 (95%CI 67.5, 80.1) [27.0, 95%CI 24.6, 29.4] in early starters and 68.7 (95%CI 64.1, 73.4) [25.1, 95%CI 23.2, 26.9] in the late-starter group.

A difference between the 2 groups was observed in the following items of the scale: standing on heels (p=0.002) jumping (p=0.004), hopping (p=0.001), lifting head (p=0.009), standing to sit (p=0.008) and the 10 metre run (p=0.05). The mean SD BMI for early starters and late starters showed no statistical difference at 7 years (p=0.2).

Figure 15. Linearized NSAA total score in DMD boys <7 years of age

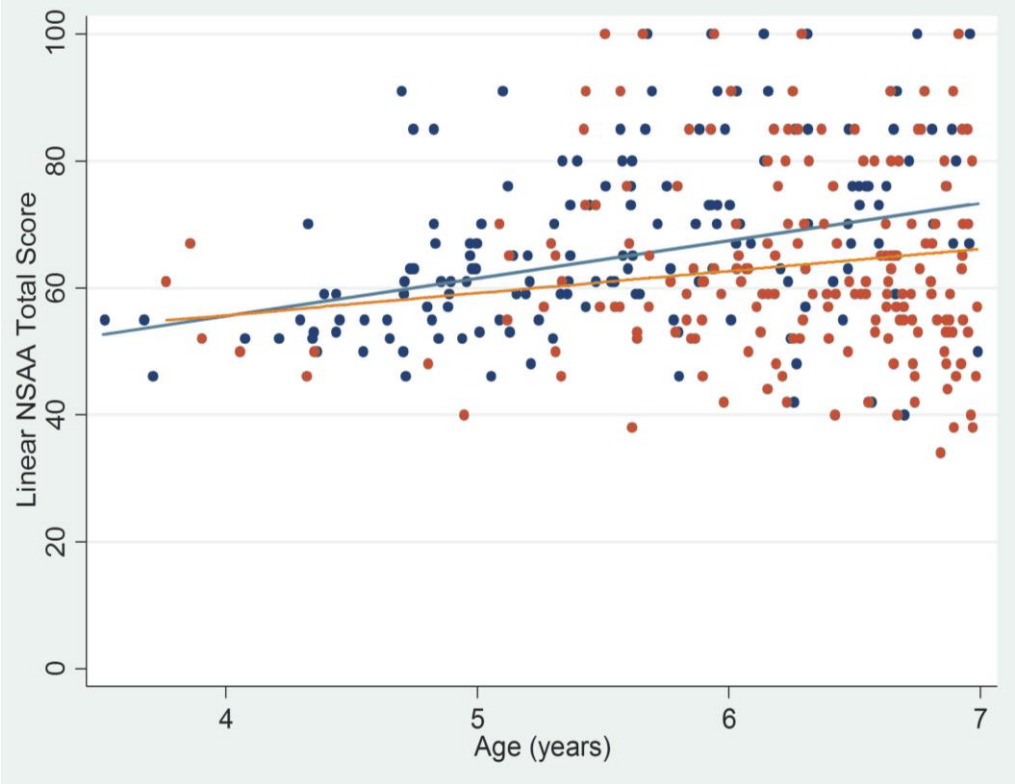


Figure 15. NorthStar ambulatory assessment in DMD boys < 7 years of age. The interaction coefficient between 78 DMD boys who started daily or intermittent GC before the age of 5 (in blue) with 163 boys who started steroids between ages 5 and 6.5 (in orange) was -2.7, (95%CI, -6.8, 1.3, p=0.2) [raw NSAA:-1.3, 95%CI -3.0, 0.3], p = 0.1 favouring early starters. By age 7, the mean total NSAA was 73.8 (95%CI 67.5, 80.1) in early starters and 68.7 (95%CI 64.1, 73.4) in the late starters (p<0.01) [raw NSAA: [27.0, 95%CI 24.6, 29.4 in early starters and 25.1, 95%CI 23.2, 26.9 in late starters].

NorthStar ambulatory assessment in UK and Italian skippable populations

For this sub-analysis we included 395 DMD (ie: 223 UK +172 Italian) boys meeting the age/motor function and GC requirement for inclusion criteria in most of on-going clinical trials as described in the methods above. The whole population showed a decline of 5 units (95% CI -6.5, -3.5; p <0.001) at 12 months and 13.8 (95% CI:-15.7, -12.0; p <0.001) at 24 months. When compared to the whole DMD population, boys skippable for exon 44 (n=27) and 46 (n=34) showed a slower decline, which became significant at 24 months follow-up (table 11, figure 16). The interaction coefficient for the population skippable for exon 44 was 2.5 (95% CI -3.2, 8.3) at 1 year (p=0.39) and 9.1 (95% CI 2.3, 15.9) at 24 months (p<0.001), suggesting that over the course of 2 years boys with exon 44 skippable deletions lost 9 points less on the transformed NSAA scale than the remaining DMD population (table 11a, figure 16). Similarly, boys skippable for exon 46 showed a slower decline at 24 months with an interaction coefficient of 8.8 (95% CI 2.6, 15.1; p<0.01). On the other hand, the negative interaction coefficient for the population skippable for exon 53 (n=41) was suggestive of an overall more rapid decline: -6.5 (95%CI -11.2, -1.7; p<0.01) at 12 months and – 14.2 (95%CI -19.9, -8.5; p<0.001) at 24 months (table 11a, figure 16). Similarly boys skippable for exon 51 (n=61) showed an increased loss of 5.7 linearized NSAA units at 2 years (interaction coefficient -5.7, p=0.02). We report the raw NSAA scores and interaction coefficients in table 11b. All the above comparisons are adjusted for treatment and age at follow-up. I found no significant difference among the remaining populations.

Table 11. Linearized and raw NSAA total score

A

	Linearized NSAA 12 months	**Linearized NSAA 12 + 24 months**
ALL DMD (n=395)	-5.0 (-6.5,-3.5) p<0.001	-13.8 (-15.7,-12.0) p<0.001
Skip 44 (n=27)	2.5 (-3.2, 8.3) (p=0.39)	9.1 (2.3, 15.9) (p<0.001)**
Skip 45 (n=31)	1.8 (-3.6, 7.4) (p=0.50)	-3.2 (-9.7, 3.5) (p=0.35)
Skip 46 (n=34)	2.8 (-2.4, 8.0) (p=0.29)	8.8 (2.6, 15.1) (p<0.01)*
Skip 50 (n=8)	4.3 (-6.6, 15.2) (p=0.44)	8.3 -4.0, 20.7) (p=0.19)
Skip 51 (n=61)	-2.5 (-6.7, 1.8) (p=025)	-5.7 (-10.6, -0.9) (p=0.02)*
Skip 52 (n=9)	1.0 (-6.7, 8.8) (p=0.79)	9.2 (0.4, 18.0) (p=0.04)*
Skip 53 (n=41)	-6.5 (-11.2, -1.7) (p<0.01)*	-14.2 (-19.9, -8.5) (p<0.001)**

B

	NSAA 12 months	**NSAA 12 + 24 months**
ALL DMD (n=395)	-2.1 (-2.7,-1.5) p<0.001	-5.8 (-6.5,-5.1) p<0.001
Skip 44 (n=27)	1.3 (-0.9, 3.5) (p=0.25)	3.9 (1.3, 6.5) (p<0.01)*
Skip 45 (n=31)	0.3 (-1.8, 2.5) (p=0.75)	-0.6 (-3.2, 1.9) (p=0.6)
Skip 46 (n=34)	1.3 (-0.6, 3.3) (p=0.19)	3.5 (1.1, 5.9) (p<0.01)*
Skip 50 (n=8)	1.9 (-2.2, 6.1) (p=0.37)	3.0(-1.8, 7.7) (p=0.22)
Skip 51 (n=61)	-1.0 (-2.6, 0.6) (p=022)	-2.4 (-4.2, -0.5) (p=0.01)*
Skip 52 (n=9)	0.4 (-2.6, 3.3) (p=0.80)	3.0 (-0.4, 6.4) (p=0.08)
Skip 53 (n=41)	-2.0 (-3.8, -0.1) (p=0.04)*	-4.5 (-6.7, -2.3) (p<0.001)**

Table 11. Linearized (a) and raw (b) NSAA total score: interaction coefficients for skippable genotypes at 12 and 24 months**.** The overall slope of decline is described for the overall DMD population at 12 months and 24 months. Skippable genotypes are compared with all DMD, at 1 year and 2 years. Interactions coefficients (p value) suggest that boys skippable for exon 44 and 46 decline at a slower rate over the course of 2 years, while boys skippable for exon 51 and 53 decline faster.

Figure 16. Mean linearized NSAA total score mean and change from baseline for skippable genotypes at 12 and 24 months

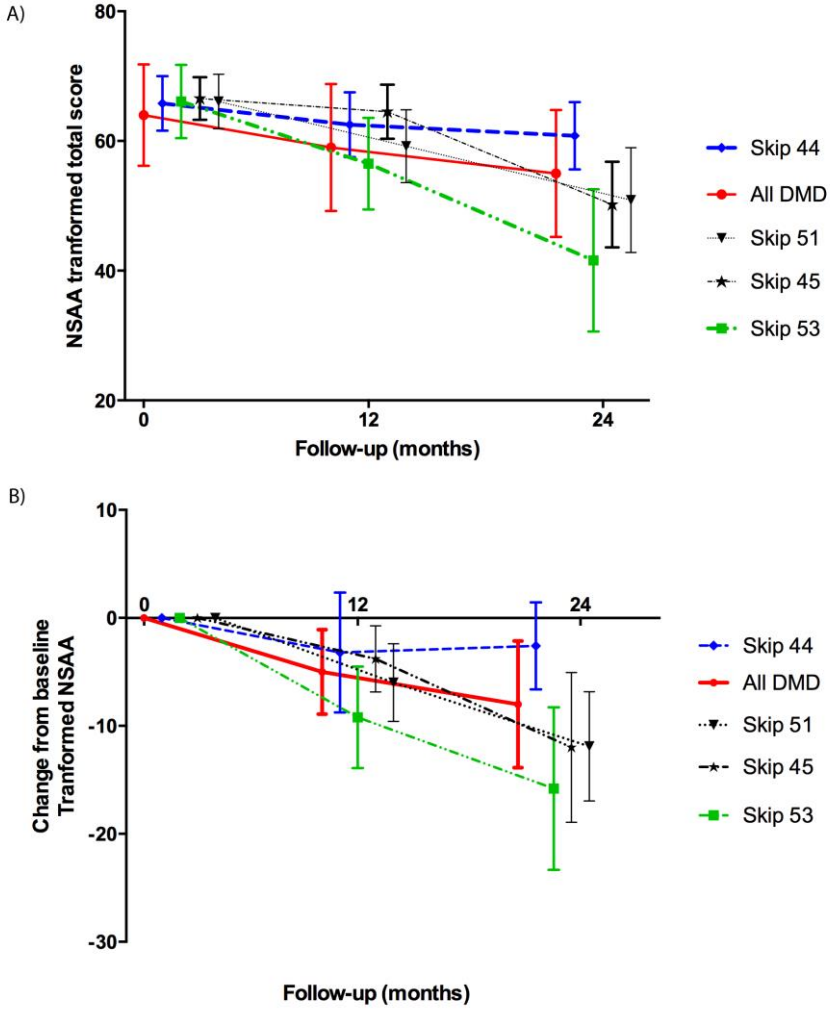


Figure 16: Mean (95% CI) Linearised NSAA total score mean (A) and change from baseline (B) for skippable genotypes at 12 and 24 months. The overall slope of decline over 24 months is described in DMD boys > 5 years of age, with a minimum NSAA of 52/100 [raw NSAA: 17/34], and at least 3 months on glucocorticoids at baseline.

Discussion

In this study I addressed a number of important issues regarding the progression of DMD, which inform patients/families, and the design and interpretation of experimental therapies.

It was recently reported on the general characteristics of children followed in the UK NorthStar clinical network.[35] In the current study I described the effect of age on disease progression on linearized NSAA scale; I explored the effect of initiating GC at a younger age than the current guidelines; in addition, I assessed the possible influence of *DMD* genotype on disease progression by gathering data from a very large group of UK and Italian DMD boys stratified by genomic mutations.

The benefit of the linearized scores is that the change score means the same across the breadth of the scale. Whereas with the raw score a drop of several points at a mid-level of ability might actually mean a small loss of function (difficulty getting on and off a small step but still independently) and a drop of one point at either end of the scale might suddenly mean loss of independence in rising from the floor or loss of ability to run.

To characterise the disease on linearized NSAA, through the collaboration of the UK NorthStar clinical network, I reported the course of DMD in >500 glucocorticoid-treated UK boys. I described the slope of progression after age 7, when motor function starts to decline. With an average linearized NSAA score of 73 at age 7 [=27/34 in the raw scale], I observed that the overall rate of decline on the transformed NSAA scale is 8 linearized units/year. Recent data suggest that in the linearized NSAA scale ~10 units are considered to capture a significant clinically meaningful change, irrespective of the scores along the scale that drive such a loss.[165] This loss can mean the loss of the ability to stand on one leg or get

up from the floor independently. Therefore the linearized NSAA can reliably support clinical trials as a secondary outcome measure for ambulant DMD, with the potential to capture either stabilization from the predicted 8-unit loss, or potentially even an improvement from baseline during the course of a one-year study. When conducting clinical trials for ambulant boys, one possible outcome is that the boys may lose ambulation during the course of the study, affecting the overall interpretation of the study. From our cohort of NorthStar-registered individuals, we found that in the 137 UK boys who lost ambulation between 9.5 and 16 years, the median age was 13 years (95% CI 12.1, 13.5). However, as age alone cannot serve as a predictor of LOA, I also reported the estimated mean linearized NSAA score at 12 and 24 months prior to LOA (i.e. at ages 12 and 11 years respectively), which in our database were 34 and 42 units [approximately 9 and 13 in the raw scale respectively]. When selecting inclusion criteria for clinical trials in ambulant boys, a baseline total linearized NSAA score of 50 (~16 of the raw scale) would therefore give reassurance that the boys are very unlikely to lose ambulation over the course of a 2-year study. Mazzone *et al.* reported that in their Italian cohort followed for 24 months, the equivalent assurance was given by performing 330 metres in the 6MWD.[159] Only 2% of the boys, who at baseline walked at least 330 metres, lost ambulation in the subsequent 2 years. Equally in their cohort of boys approximately 98% of boys with a NSAA score of 17/34 (~52 units in the linearized NSAA) were ambulant after 2 years, confirming our observation.[159]

If recruiting older DMD patients in clinical trials poses the problem that they may lose ambulation during the course of the study, recruiting younger individuals poses the opposite problem: a number of DMD patients improve their motor function up to the age 7,[35, 113, 160, 161] thereby complicating the

interpretation of a potential drug effect against the natural disease course. In our study we report a functional gain of ~3 units/year between 4-7 years [~1.5 raw NSAA], with a mean NSAA score at age 7 of 73 (p=0.02) [=27/34]. We further report an improvement of 3 additional linearized units/year in 36 boys who had started GC <5 years (mean age: 3.4 years) when compared to boys who started GC >5, with a difference of 10 units by age 7 (p<0.01) but no difference in SD BMI at this early phase of therapy. We lack sufficient longitudinal data to conclude that this initial benefit is sustained also during the decline phase and that age of LOA is further postponed, although data from smaller cohorts also appear to substantiate an increased efficacy of early GC initiation.[135] Additionally we lack sufficient longitudinal data to show if the cumulative GC therapy has an enhanced detrimental effect on the side effect profile. However, despite the limitation of our relatively small cohort (n=78), the positive impact of early initiation of GC therapy is of interest and demands future exploration. Furthermore, our analysis provides an insight on the motor function in this age group of patients, who are likely candidates for future clinical trials.

As part of our study I also explored progression of disease in mutations amenable to genetic therapies. A number of current experimental approaches for DMD are mutation specific,[78, 80, 152, 153, 158, 167] emphasizing the importance of investigating, within the disease spectrum, possible existing variations in the natural course of each genetic subtype, which could impact on clinical trials. Exon skipping by antisense oligomer is now in Phase III studies for exon 51; molecules targeting the exons 44, 45,and 53 are undergoing early clinical studies. In this study, I observed that individuals with duplications deteriorate minimally slower (~1 unit/year) when compared to individuals with point mutations and deletions, however this did not meet significance. In a sub-study, we combined

the Italian and UK datasets, to focus the analysis on skippable deletions and reproduce the setting of a clinical trial. I observed that boys skippable for exon 53 do progress more rapidly losing additional 14 units over 2 years [~4-5 raw units], when compared to the whole DMD population (p=0.001). Similarly, individuals skippable for exon 51 declined faster loosing additional 5 units [2.5 raw units] (p=0.02). In contrast, boys with deletions skippable for exon 44 showed a less rapid decline measured as 9 linearized units over 24 months (p<0.001) [~4 raw units]. Within the spectrum of severity of DMD, variability in phenotype among skippable deletions has been previously reported. Pane *et al.* observed that boys skippable for exon 44 had better baseline results in the 6MWD and less drastic changes over the course of one year when compared to boys skippable for exon 53.[168] Despite a similar trend, their results at one year did not reach statistical significance. The results of the present study are consistent with this observation, as we could not detect a significant difference at 12, but only at 24 months. Similarly, Servais *et al*.[169] reported that patients treatable by exon 53 skipping have a more severe phenotype when upper limb function was assessed in a cohort of 14 DMD; additionally loss of ambulation had occurred one year earlier in 90 boys who were 53 skippable when compared to 400 other DMD boys. Residual dystrophin level can partially explain such a phenomenon. Recent studies showed that a larger presence of revertant fibres and residual dystrophin expression, which may favour a better outcome, is found in patients with mutations in the region of exons 45-47,[162, 163] when compared to levels of dystrophin found in patients with mutations in the region of exon 47-55.[170] It is possible to attribute these higher levels of residual dystrophin proteins to spontaneous exon 44 skipping when surrounding exons are deleted.[171] These "leaky" mutations can thus present with more variable phenotypes, which

highlight the limitation of an exclusively genetic diagnosis/prognosis and the value of accurate muscle pathology.

Residual dystrophin levels can therefore play a role as a weak modifier of DMD progression, although further exploration in larger cohorts should be performed in order to power studies precisely. The size effect of these differences is small and in practice negligible for studies lasting 1 year or less, but should be considered for longer duration studies. This might be particularly important in clinical trials where a treatment arm is compared to natural history data or a subset of patients. In particular our data would discourage the recruitment of patients with deletions skippable for exon 44 as a comparator group for patients with 53 skippable deletions, and vice versa. Moreover, other disease modifiers are increasingly being reported in DMD, including polymorphisms in SPP1[172, 173] and LTBP4.[174] This latter gene was very recently independently validated in separate DMD patient populations.[175] When interpreting response to treatment the combination of disease modifiers should therefore be accounted for.

Our study has some limitations: incomplete and partially missing data, adjusted for in the statistical analysis; patients of the same genotype sub-group were on different steroid regimens (i.e. daily or intermittent), also adjusted for in the analysis; although this study included the largest sample size ever studied when correlating phenotype to genotype, the number of patients included for the analysis remains relatively small; finally, also the early starters used different GC regimens and long-term effects, such as LOA and adverse events could not be evaluated because the follow-up is still ongoing.

Despite its limitation, this study describes up-to date natural history data of the linearized NSAA scale in a large cohort of DMD boys in the UK and Italy. The NSAA is currently included in clinical trials as a secondary outcome measure.

Increased knowledge of the natural course of the disorder and its covariates (ie: age at starting GC and gene mutations), which can influence sensitivity of clinical trials, will help for the design of future studies and interpretation of their results.

CHAPTER 4: THE NEURO-PSYCHIATRIC PROFILE OF DMD

The work contained in this chapter has given rise to the following publication:
Ricotti V, Mandy WP,Scoto M, Pane M, Deconinck N,Messina S, Mercuri E, Skuse DH, Muntoni F. *Neurodevelopmental, emotional and behavioural problems in Duchenne muscular dystrophy in relation to underlying dystrophin gene mutations.* Dev Med Child Neurol. 2015 Sep 14. [Epub ahead of print] PMID:26365034

My contribution to this work consisted of the following:
I oversaw the design of the study. I led recruitment of patients into this study and contributed to data collection. In collaboration with William Mandy I analysed the data. I wrote the first draft of the manuscript, and contributed to the revision of the manuscript.

Dystrophin and the brain

Earlier clinical description of DMD, including descriptions by Duchenne himself in 1868 and by Gowers in 1879 had reported that a proportion of young males affected by DMD had some degree of intellectual impairment, however for almost a century, the neuropsychiatric aspects of DMD have been overlooked and poorly understood until more recently.

Some studies in the 1950s had found that the incidence of cognitive impairment in DMD was the same as that among the general paediatric populations, [176, 177] others explained the intellectual difficulties as a consequence of the chronic disability alone or in combination with low-socio economic status. [178, 179]

However, more research started in the 1960s confirming the initial observations and establishing substantial evidence that DMD patients do suffer from decreased cognitive functions. Table 12 summarises the findings of these key initial studies.[180-185] Of interest, Posser *et al.* also examined the IQ of 31 normal brothers and compared it with the IQ of their affected DMD siblings. A significant difference was reported with the normal siblings having a mean IQ in the normal range (110.4 ±13.0 SD).[185]

Table 12. Early studies on cognitive function in DMD

Study reference	No. of subjects	Age of subjects	Mean IQ (ranges)	IQ< 75
Allen et al 1960	30	2-23	82 (14-117)	30%
Worden et al 1962	38	4-27	83 (46-134)	29%
Shoer 1964	28	5-28	n.a.	n.a.
Dubowitz 1965	27	8-16	68 (42-118)	70%
Zellweger et al. 1965	42	3-16	83 (42-131)	33%
Zellweger et al.1967	48	2-8	83 (48-127)	31%
Prosser et al. 1969	52	2-18	87 (51-113)	30%

Serial testing of a sub-set of subjects also suggested that cognitive impairment, unlike motor deterioration, does not appear to be progressive. [184, 185] In the early 1980s it was further recognised that in addition to cognitive impairment, DMD boys also suffer from a high rate of emotional disorders.[186]

Only recently, there has been a substantial body of evidence showing the wide spectrum of neuropsychiatric disturbances in DMD and its growing recognition as a direct result of disruption of CNS dystrophin protein products.

The large *dystrophin* gene (2.5 million bp) contains 79 exons plus seven promoters, each linked to a unique first exon. These tightly regulated internal promoters generate a range of different protein isoforms, with diverse expression in tissues. Mutations in the 5' end of the gene (i.e. mutations from exon 1 to 31) only affect the 3 longest isoforms Dp427M, Dp427C, and Dp427P, expressed in skeletal and cardiac muscles, in the neurons in cortex, and in cerebellar Purkinje cells respectively. However, mutations progressively further along the gene affect more and more isoforms. Mutations in between exons 31-44, in addition to disrupting expression of the long isoforms, will also disrupt Dp260 (expressed mostly in the retina); mutation in between exon 45- 62 will in addition disrupt both Dp140 (expressed in the brain and kidneys), Dp116 (expressed in Schwann cells), while the rare mutations downstream of exon 63 will affect all dystrophin products including the shortest Dp71 (most abundant brain isoform) (figure 17).[18]

Figure 17. Genomic organisation of the dystrophin gene

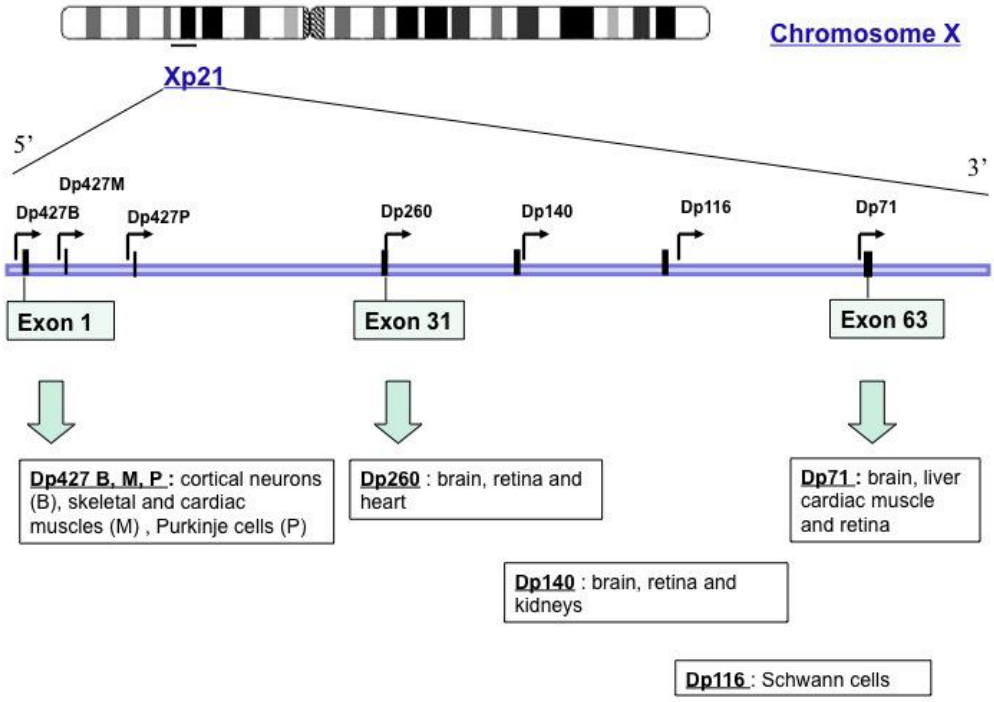


Figure 17. Genomic organisation of the dystrophin gene. The various promoters along the gene are illustrated. Mutations along the gene progressively disrupt the various protein products, the expression of which varies in different tissues, including the brain

(Adapted from Muntoni et al, Lancet Neurol. 2003)

In muscle dystrophin is a large sarcolemmal protein, part of the dystrophin-associated protein complex, connecting the cytoskeleton to the extracellular matrix.[187] A major function of dystrophin in muscle is to protect muscle fibres against the mechanical forces of contraction, thus its absence renders muscle fibres susceptible to stretch-induced damage and necrosis.[188] Dystrophin has also been assigned a signalling role; while some of these activities are relevant for skeletal muscle function (e.g. modulation of adrenergic tone by nNOS)[26], others are essential for dystrophin function in organs such as brain and retina. [26, 189-191] The role of dystrophin isoforms in the brain remains largely unclear; nevertheless it is recognised that the brain is affected by the lack of dystrophin and notably that mutations disrupting the brain isoforms Dp140 and Dp71 are more frequently associated with lower IQ scores.[66-68, 73] Furthermore, CNS involvement in DMD goes beyond disability arising from impaired intellectual development. A number of recent studies showed that DMD boys present with symptoms of neurodevelopmental disorders, with higher prevalence than in the general paediatric population including: Autistic Spectrum Disorder (ASD) reported between 3-19%[69-71, 73]; Attention Deficit and Hyperactivity Disorder (ADHD) between 12-31%[70, 72, 73]; and Obsessive Compulsive Disorder (OCD) in up to 5%.[70] In accordance with what was reported about cognitive function, higher prevalence of ADHD has been reported in association with mutations predicted to affect Dp140 and Dp71.[72]

Autistic Spectrum Disorder (ASD) describes a range of conditions classified as neurodevelopmental disorders in the fifth revision of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5). These conditions are characterized by social deficits and communication difficulties, stereotyped or repetitive behaviours and interests,

sensory issues, and in some cases, cognitive delays. The general population prevalence is estimated to be about 1-6%.[192]

Attention Deficit and Hyperactivity Disorder (ADHD) is a neurodevelopmental disorder, in which there are significant problems with executive functions (e.g. attention control and inhibitory control) that cause attention deficits, hyperactivity, or impulsiveness that is not appropriate for a person's age. These symptoms must begin by age six to twelve and persist for more than six months for a diagnosis to be made. It affects about 6–11% of children.[193]

An individual with inattention may have some (or al) of the following symptoms:

- Be easily distracted
- Have difficulty maintaining focus on one task
- Become bored with a task after only a few minutes
- Have difficulty focusing attention
- Have trouble completing or turning in homework assignments
- Not seem to listen when spoken to
- Daydream
- Have difficulty processing information as quickly and accurately as others
- Struggle to follow instructions

An individual with hyperactivity may have some (or all) of the following symptoms:

- Fidget and squirm in their seats
- Talk nonstop
- Dash around, touching or playing with anything and everything in sight

- Have trouble sitting still during dinner, school, and story time
- Be constantly in motion
- Have difficulty doing quiet tasks or activities

Emotional and behavioural disorders (EBD) are a broad category, which group together as a range of more specific perceived difficulties of children and adolescents. These are divided in internalising problems, such as depression and anxiety; and externalising problems such as aggression, under-control and acting out. It is very difficult to estimate their prevalence, however it is estimated that at least 10% of children and adolescents are affected at some stage of their life from childhood to adolescence.[194]

Finally, **Obsessive–compulsive disorder (OCD)** is an anxiety disorder characterized by intrusive thoughts that produce uneasiness, apprehension, fear or worry (obsessions), repetitive behaviours aimed at reducing the associated anxiety (compulsions), or a combination of such obsessions and compulsions. The estimated prevalence is between 1 to 3% of children and adults.[195]

A study on the neurodevelopmental, emotional and behavioural problems in Duchenne muscular dystrophy in relation to underlying dystrophin gene mutations

In this multicentre international study, I aimed to elucidate the neuropsychiatric phenotype of DMD by using a comprehensive battery of standardised, well-validated neurodevelopment assessments. In so doing we aimed to offer the most comprehensive picture to date of the neuropsychiatric elements of DMD, by capturing with parent reporting a wide array of CNS characteristics including: 1)

intellectual difficulties (ID), 2) neurodevelopmental disorders (i.e. ASD, ADHD,) 3) emotional behavioural problems such as internalising and externalising behaviours. We aimed to ascertain how these different characteristics cluster in the same individual, exploring the possibility of a "*DMD neuropsychiatric syndrome*". Finally, we aimed to learn about the association between the neuropsychiatric profile and genotype of DMD, in particular referring to those regions of interest disrupting the different dystrophin isoforms.

Methods

Patient population

Families attending the neuromuscular departments in three counties (UK, Italy and Belgium) and four centres were recruited over 12 months. Inclusion criteria were as follows: (1) DMD boys with proven mutation in the *dystrophin* gene. Mutations were classified according to the Leiden Muscular Dystrophy database (http://www.dmd.nl): (2) aged between 5 and 16 years; and (3) willing to take part into the study and with good understanding of the language spoken in the country of the assessment.

Ethic approval was obtained by all participating centres.

Measures

Social Communication Disorder Checklist

Families attending the neuromuscular outpatient department were asked to complete a validated, 12-item measure, the Social Communication Disorder Checklist (SCDC), which is highly sensitive to neurodevelopmental disorders

especially in the social communication domain. [196, 197] The SCDC is a 3-point Likert scale, with each item scoring 0, 1 or 2, with higher scores indicating greater symptom severity. A total score of ≥8 is indicative of likely neurodevelopmental disturbance, whilst scores >15 are strongly suggestive of ASD.

Cognitive assessment

Intelligence was measured, where possible (n=92, 71%), with the Wechsler Intelligence Scales for Children- Fourth Edition (WISC-IV).[198] A truncated version of the WISC-IV was administered (Vocabulary, Similarities, Matrix Reasoning, Block Design, Digit Span and Letter-Number Sequencing) to reduce participant burden. The Digit Span and Letter Number Sequencing subtests were used to produce a Working Memory Index (WMI) score; Similarities and Vocabulary subtests produced a Verbal Comprehension Index (VCI) score; and the Matrix Reasoning and Block Design subtests generated a Perceptual Reasoning Index (PRI) score. General intelligence was measured using the WISC-IV's General Ability Index (GAI) score. This is a measure of overall intellectual ability that is particularly suitable for boys with DMD as, compared to the full-scale IQ score of the WISC-IV, it is less influenced by processing speed, working memory deficits and motor problems, which are common in this population.[199] In addition, three boys (2%) who were aged five when they joined the study, and so were too young for the WISC-IV, were assessed using the Wechsler Preschool and Primary Scale of Intelligence – Third Edition (WPPSI-III).[200]

In boys reluctant to complete a Wechsler intelligence test or those who were non-verbal (n=20, 15%), general intelligence was assessed with the Raven's Coloured Progressive Matrices (RCPM).[201] RCPM is a widely used, well-normed cognitive

assessment for children aged 5-11. In the current study raw scores were converted to IQ scores with a mean of 100 and a SD of 15.[202] Boys with a GAI or RCPM IQ score equal to or less than 70 were categorized as being in the ID range.[203] An additional 15 boys (12% of overall sample) were able to complete neither a WISC-IV, WPPSI-III nor RCPM, due to being non-verbal and having developmental delay. After inspection of their clinical notes, they were all estimated to have an ID, based on reports of their language, functioning and presentation in clinic.

Neurodevelopmental assessments

ASD was assessed with the Developmental, Diagnostic and Dimensional Interview short version (3Di-sv).[204] The 3Di-sv is a semi-structured, parent-report, diagnostic interview comprised of 53 items. It has high inter-rater reliability and test-retest reliability coefficients.[204] The clinical cut-off scores on the 3Di-sv indexes are reciprocal social interaction (RSI) impairment=11.5, repetitive and stereotyped behaviour (RSB)=5; and communication impairment (CI)=8. Children who score above the cut-off point on the RSI scale and either the RSB scale and/or CI scale are categorised as meeting criteria for ASD.

In addition, parents completed the *Conners' Parent Rating Scale (Conners 3)*, The short version of the Conners' Parent Rating Scale is a 45-item questionnaire addressing issues such as: aggression, learning problems, inattention, hyperactivity/impulsivity, executive functioning and peer relations. Children who had t-scores in the abnormal range (t<65) on the hyperactivity and/or inattention indexes were categorized as meeting criteria for ADHD by parent report.[205] In the UK cohort alone, also teachers were asked to complete the

Conners' Teacher Rating Scale (Conners 3), and return the questionnaire by pre-stamped envelope.

Emotional behavioural problems

Parents completed the Child Behavioural Check List (CBCL). The CBCL is a parent-report measure, with scales measuring internalizing symptoms (anxiety and depression), and externalizing symptoms (aggression and hyperactivity). It is normed for use with young people between the ages of 4 and 18 years. In line with the CBCL manual, internalising, externalising and total t-scores greater than or equal to 63 were deemed to be in the clinical range. [206]

Statistical methods

To compare the SCDC mean score of the current sample with published population norms, a one sample T-test was used. One-way ANOVA was used to compare mean scores on continuous measures in the three genotype-defined sub groups of the sample. Post hoc analyses were conducted using Gabriel's procedure, which has been designed to accommodate for situations when sample sizes are very different. [207] Fisher's exact tests were used to make group comparisons for categorical variables. Paired samples t-test was used to compare parents and teacher's scores where available.

Results

We recruited 130 DMD boys in 4 centres: UK (n=73); Italy, (Rome n=29; Messina n=16); Belgium (n=12). Mean age was 9.8 (range: 5-16 years) including both ambulant and non-ambulant subjects. All boys were treated according to the

standards of care for DMD.[32] The overall grouping for genotype was as follows: 41 patients with mutations upstream of exon 30; 75 boys with mutations in between exons 31-62, and 14 boys with mutations downstream of exon 63. There was no difference in mean age between the genotype sub-groups.

Social Communication Disorder Checklist (SCDC)

The mean total SCDC score for 130 DMD boys was 7.9 (95% CI 6.8, 8.9), which was significantly higher ($p<0.001$) when compared to the mean score of normal controls (2.9, 95% CI 2.2, 3.6) previously reported[208], indicating greater levels of impairment in this DMD sample. The boys with mutations predicted to disrupt all short/brain dystrophin isoforms, including Dp71 (i.e. with mutations downstream exon 63), had a total mean score of 11.7 (95% CI 8.5, 15.4); boys with mutation disrupting Dp260, Dp140, Dp116 (i.e. between exons 31-62) had a mean score of 7.3 (95% CI 5.9, 8.6); and boys with Dp427 mutations (i.e. upstream exon 30) had a mean score of 7.6 (95% CI 5.8, 9.3). ANOVA confirmed a significant group effect ($p=0.045$). Post hoc testing using the Gabriel's procedure showed that this reflected higher scores of boys with mutations downstream of exon 63, compared to those with mutations upstream of exon 30 ($p=0.024$). Also, we observed that boys with mutations downstream of exon 63 had higher SCDC scores than boys with mutations between exons 31 and 62, although these findings were of clinical importance, they did not reach statistical significance ($p=0.071$).

Cognitive Function

We observed that 34 out of 130 DMD boys (26%) were estimated to have an intellectual disability (ID). For 19 of these ID status was assigned on the basis of

an IQ score below 70 (table 2), and for 15 ID was estimated from clinical notes as they presented with too severe a language and developmental delay to complete an IQ test. WMI was on average strongly affected (table 13). Post hoc testing using the Gabriel's procedure showed group effect was seen (p=0.011), with a more profound ID associated with mutations downstream exon 63 (table 13). Furthermore, nine out of 14 individuals with mutations affecting Dp71 (i.e. downstream exon 63) had intellectual disability (64%) compared to 25% (19 out of 75) of boys with mutations disrupting Dp260, Dp140, Dp116, and 15% (6 out of 41) of boys with mutation disrupting only the long dystrophin isoforms Dp427 (table 14).

Neurodevelopmental disorders

Of the 130 subjects, 87 completed the full assessment protocol (UK n=62, Italy n=13, Brussels n=12). There was no bias based on clinical presentation in selecting these 87 boys: the primary reason why the remaining boys did not undergo the full assessment was due to time restraint during the routine clinical appointment. The 87 boys were representative of the whole cohort: they did not differ significantly from the remaining individuals in relation to age, IQ, SCDC score and genotype (all p's >0.09). The genotype grouping was as follows: 26 subjects with mutations upstream of exon 30; 54 boys with mutations between exons 31-62, and 7 boys with mutations downstream of exon 63.

In this entire cohort, we observed a high prevalence of individuals who were above the threshold scores on the standardised parent-report instruments employed. A fifth (21%, n=18) of the boys scored in the ASD range, 24% (n= 21) showed clinically significant hyperactivity and 44% (n=38) had severe difficulties

with inattention (table 14). The mean raw scores are also reported (table 13). With the exception of hyperactivity, where a group difference was observed, with mutations at the 3'end of the gene being more severely affected (p=0.039), we did not find any statistical difference between groups. However, of clinical interest, a higher proportion of boys with mutations affecting the shorter isoforms met the threshold scores for neurodevelopmental disorders as opposed to boys with mutations only affecting Dp427 (table 14).

Conners' 3 teachers

Conners' 3 (T) data was obtained for 41 out of 52 boys recruited in the UK (66%). Teachers gave 15 out of 41 boys (37%) scores that indicated ADHD symptomology. On paired samples correlations both the inattention (r= 0.590; p<0.001) and hyperactivity (r= 0.601; p <0.001) index scores of the parent and teacher measures were significantly correlated. There was no significant difference between the mean scores reported by parents and teachers on the hyperactivity index; however, a paired samples t-test revealed that parents reported a significantly higher mean score on the inattention index (mean= 62.61; SD =13.28) than teachers (mean = 55.78; SD = 10.73), t = 6.83, p=0.001.

Emotional behavioural problems

As is shown in table 14, emotional and behavioural problems were highly prevalent in our cohort of 87 DMD boys. Around a quarter (n=21) had scores in the clinical range for internalising problems (i.e. anxious, depressive, and over-controlled); and approximately one in six (n=13) scored in the clinical range for externalising behavioural problems (i.e. aggressive, hyperactive, noncompliant, and under-controlled.). On the CBCL's total problems scale, 15 boys (17%, 95 CI

10,26) scored above the conventional clinical threshold (t-score ≥ 63), and 13 (15%, 95% CI 8,23) scored above a more stringent threshold (t-score ≥ 67), employed in previous research using the CBCL in a group of boys with DMD.[209] Unlike ID, and neurodevelopmental disorders, internalising and externalising problems were not related to genotype: they were common in the boys with mutations at the 3'end of the gene, and amongst boys with mutations predicted to affect the Dp427 alone, so they inversely correlated with ID (table 14).

Table 13 Raw scores for the 3Di-sv, Conners 3, CBCL and general intelligence in the total population and according to genotype sub-groups

Assessment		Mean score (95% CI)				P-values	
		Upstream exon 30	**Exon 31 -62**	**Down-stream exon 63**	**TOTAL**	**p-value**	**Post-hoc**
Cognitive function[$]	General Ability Index	96.0 (87.9, 104.1)	91.7 (86.7, 96.7)	74.8 (62.7, 86.9)	91.8 (87.7, 95.8)	0.042*	31 > 63
	Working Memory Index	86.6 (78.2, 94.9)	81.7 (75.8, 87,5)	57,5 (24,4, 90.7)	81.3 (76.2, 86.3)	0.011*	31,31-62 > 63
3Di-sv	Social	7.6 (6.1, 9.2)	8.8 (7.3, 10.3)	10.5 (4.9, 16.0)	8.6 (7.5, 9.7)	0.396	
	Communication	7.8 (5.9, 9.7)	7.76 (6.2, 9.2)	10.20 (4.35, 16.1)	6.6 (6.9, 9.7)	0.516	
	Repetitive stereotypical behaviour	4.1 (2.8, 5.7)	3.2 (2.4, 4.0)	4.4 (1.5, 7.6)	3.6 (2.8, 4.2)	0.387	
Conners-3 (Parents)	Inattentive	62.1 (57.3, 66.9)	59.5 (57.2, 67)	63.6 (47.5, 79,6)	60.6 (57.1, 64.1)	0.706	
	Hyper-activity	53.4 (50.1, 56.9)	53.4 (49.5, 58.3)	63.1 (52.6, 73.7)	54.5 (51.5, 57.5)	0.240	
CBCL	Internalising Problems	9.4 (6.3, 12.6)	8 (7.3, 11.7)	6.3 (3.5, 11.1)	9.5 (7.8, 11.3)	0.998	
	Externalising Problems	8.31 (5.2, 11.3)	9.98 (7.4, 12.5)	8.7 (1.5, 15.9)	8.6 (7.5, 11.2)	0.708	

Table 13: Raw scores for the 3Di-sv, Conners 3, CBCL and general intelligence (WISC-IV/RCPM) in the total population and according to genotype sub-groups. [$]Upstream exon 30 n=25; mutations between exon 31 -62 n=52; Downstream exon 63 n=6. Non-verbal patients were not included. ANOVA, p-values <0.05 were considered significant*.

Table 14 Prevalence of cognitive function, neurodevelopmental disorders and emotional behavioural problems for all mutations and according to genotype

		Genotype subgroups (mutations)			Total, n (%, 95% CI)	**P value**
		Upstream exon 30 (Dp427)	Exon 31 -62 (Dp260, Dp140, Dp116)	Down-stream exon 63 (Dp71)		
Cognitive function (n=130)						
	Intellectual disability	6/41 (15%, 7, 29)	19/75 (25%: 17, 37)	9/14 (64%, 36, 85)	34 /130 (26%, 19, 34)	0.002
Neurodevelopmental disorders (n=87)						
	Scores above threshold for Autistic Spectrum Disorder	4/26 (15%; 6,35)	11/54 (20%; 11,34)	3/7 (43%; 13, 79)	18/87 (21%; 13,31)	0.264
	Scores above threshold for inattention	13/26 (50%; 31, 69)	22/54 (41%; 28, 55)	3/7 (43%; 13, 79)	38/87 (44%; 33, 54)	0.753
	Scores above threshold for hyperactivity	3/26 (11%; 4,31)	14/54 (26%; 16, 39)	4/7 (57%, 21, 87)	21/87 (24%; 16, 34)	0.046
Emotional behavioural problems (n=87)						
	Scores above threshold for internalising problems	6/26 (23%, 10, 44)	14/54 (26%; 16, 39)	1/7 (14%; 2, 63)	21/87 (24%; 16, 34)	0.929
	Scores over threshold for externalising problems	2/26 (8%; 2, 27)	10/54 (19%; 10, 31)	1/7 (14%; 2, 63)	13/87 (15%; 9, 24)	0.404

Table 14. Prevalence of impaired cognitive function, neurodevelopmental disorders and emotional behavioural problems for all mutations and according to genotype (Fisher's exact tests). P-values of <0.05 were considered significant.

Clustering of neurodevelopmental, behavioural and emotional symptoms

In light of the multitude of neurodevelopmental, emotional and behavioural problems observed in our population, we looked at how many of these cluster in the same subject. We found a high prevalence of clustering of psychiatric symptoms: 37% of boys (n=32 of 87) scored in the clinical range on more than one measure of emotional, behavioural or neurodevelopmental problems (table 3). Twelve boys (14%) scored up on four or more of these measures. Compared to boys with mutations affecting only the long Dp427 isoforms (n=3, 12%), those with a mutation affecting the short isoforms (n=17, 28%,) were more than twice as likely to have clinical problems on three or more measures, although this difference was not significant (p=0.162). In table 16 I report the correlation within psychiatric comorbidities.

Despite the high prevalence of neuropsychiatric disturbances, it is also noteworthy that 32 (37%) DMD boys had no symptoms detected.

Table 15. Clustering of neurodevelopmental, emotional and behavioural problems for all mutations and according to genotype

Symptoms	Mutations between exon 1-30, n (%, 95% CI)	Mutations between exon 31 – 79, n (%, 95% CI)	All mutations, n (%, 95% CI)
0	8 (31; 16, 51%)	24 (39%; 28, 52)	32 (37%; 27, 48)
1	9 (35%; 19, 55)	14 (23%; 14, 35)	23 (26%; 18, 37)
2	6 (23%; 11, 44)	6 (10%; 4, 21)	12 (14%; 8, 23%)
3	2 (8%; 2, 27)	6 (10%; 4, 21)	8 (9%; 5, 18%)
4 plus	1 (4%; 1, 24)	11(18%, 10, 30)	12 (14%; 8, 23%)
	Total n=26	***Total n=61***	***Total n= 87***

Table 15. Clustering of neurodevelopmental, emotional and behavioural problems for all mutations and according to genotype.
These included: intellectual disability, ASD, ADHD, internalising and externalising problems. A total of 32 DMD (37%) boys across all mutations had a clustering of two or more symptoms. Boys with mutations affecting the short brain isoforms had a higher proportion of clustering of three or more symptom (28%, n=17) than boys with mutations at the 5' end of the gene (12%, n=3), however this was statistically non-significant.

Table 16. Pearson Correlation between neurodevelopmental disorders, emotional behavioural problems and intellectual disability

	1	2	3	4	5	6	7	8
1. ASD (Social Domain)	1	0.622**	0.558**	0.530**	0.506**	0.336**	0.520**	0.23*
2. ASD (Communication domain)		1	0.463**	0.364**	0.307**	0.230*	0.363**	0.31*
3. ASD (Stereotypical Behaviour)			1	0.340**	0.329**	0.227*	0.384**	0.03
4. Inattentive				1	0.670**	0.370**	0.591**	0.18
5. Hyperactive					1	0.476**	0.643**	0.05
6. Internalising problems						1	0.616**	0.09
7. Externalising problems							1	0.07
8. Intellectual disability								1

Table 16. Pearson Correlation between neurodevelopmental disorders, emotional behavioural problems and intellectual disability. *Upstream exon 30 n=25;

between exon 31 -62 n=52; Down-stream exon 63 n=6. Non-verbal patients were not included (**p=0.002; *p=0.05)

We observed a strong correlation between comorbidities, most strikingly between the social domain of ASD and externalising problems (r=0.52, p=0.002); ADHD and the social domain of ASD (r=0.5, p=0.002); internalising and externalising problems (r=0.6, p=0.002). Intelligence, where measured, did not show a strong correlation neither with neurodevelopmental disorders, nor with emotional problems.

Discussion

I reported a large multicentre and international cohort study that used well-validated parent-report measures of neurodevelopmental, behavioural and emotional difficulties to screen DMD boys with a wide spectrum of genetic mutations.

This study found a significantly elevated mean total SCDC score, suggestive of high rates of neurodevelopmental disturbance in this DMD population.[196] In relation to general intelligence, we confirmed previous findings reported in the literature: cognitive function was lower than in the general population with WMI most strongly affected.[210] [211] Including 15 boys who were non-verbal/profoundly delayed, we estimated that 26% (n=34) had ID. Based on detailed and standardised parent-report measures we observed a high prevalence of neurodevelopmental disorders: 21% of boys scored above the threshold for ASD, 24% for hyperactivity, 44% for inattention. These figures are much higher than those in the general paediatric population.[212-215] Our study fits with previous observations on the high prevalence of ADHD, reported as 32% in a cohort of Italian DMD boys assessed according to DSM-IV criteria.[72] In relation to ASD, previous studies supported an association with dystrophinopathies: Hinton and colleagues [71] reported that 19% of DMD/BMD met the criteria for ASD in the Autism Diagnostic Interview-Revised. Our findings are based on a diagnostic interview with parents; although the 3Di is a reliable, highly sensitive and specific tool for ASD, it may overestimate the diagnosis; therefore these findings need further exploration with in-depth objective assessments which include direct observation with tools such as the Autism Diagnostic Observation Schedule. However, it is undisputable that ADHD and ASD have traditionally

been an under-diagnosed co-morbidity of DMD, which is a concern given the high levels of functional impairment associated with both disorders.

Emotional behavioural problems, such as anxiety, affective disorder, and oppositional/aggressive behaviour have also been recognised in DMD.[209, 216] One study reported that on a screening behavioural questionnaire, 32% of DMD families responded with scores above the normal cut-off, when compared to 0-2% of other disabling neuromuscular disorders, including spinal muscular atrophy.[217] Only a small proportion of these DMD patients had been investigated for neurodevelopmental disorders. In our large DMD cohort, we observed that families reported also a high prevalence of internalising and externalising problems, 24% and 15% respectively. These findings reflect the high level of anxiety and mood disorders, alone or in combination, observed in clinical practice.

Although it is not uncommon for neurodevelopmental disorders to cluster together[212], in our DMD cohort we observed a high prevalence of combination of neuropsychiatric symptoms: over a third of boys presented with ≥2 co-morbidities, and 8% of DMD boys with ID (n=7) scored in the range all four disorders assessed. Such striking patterns of clustering of symptoms suggest the existence of a "*dystrophin-specific neurodevelopmental syndrome*", which in clinical practice has a significant impact on the condition and its management. In future, it will be important to further evaluate DMD with objective neuropsychiatric inventories, and to use non-DMD neurodevelopmental control group to see if there is any distinctive pattern of clustering observed in DMD.

Mean life expectancy for DMD is now in the late 20s, with many individuals surviving into the 4th and even the 5th decade of life; it is anticipated this will further shift in the new generations. Therefore, improving neuropsychological

wellbeing is of paramount importance for participation and quality of life. Intervening from early stages, during the crucial initial years of schooling, targeting specific ID and/or behavioural issues, first of all requires prompt and appropriate recognition and understanding of the disorder.

In our study we explored further a relationship between genotype (i.e. mutation location along the gene) and the neuropsychiatric profile. On the 12-item SCDC questionnaire completed by the families, we found that boys with Dp71 mutations (i.e. downstream exon 63) had overall the highest SCDC mean total score (=11.7), with more than half of the boys scoring in the abnormal range of ≥8. In relation to intelligence, confirming previous reports, [67, 218]DMD boys with mutations affecting an increasing number of shorter isoforms had a higher frequency of ID (p=0.002), with 63% of Dp71 mutations being most severely affected. WMI also showed a significant group effect (p=0.011), with the group missing the short isoform being worst affected. In relation to the detailed neurodevelopmental assessment of a subsample (n=87), our analyses were somewhat limited by the small numbers of boys with mutations affecting the 5' end of the gene, which meant we only had the statistical power to detect very large effects. Nevertheless, in common with previous findings for ADHD, we observed higher levels of hyperactivity amongst boys with the 5' mutation. Clinically, these same boys to have a higher incidence of ASD, and this will be worth investigating in larger samples.

In contrast to neurodevelopmental problems, emotional behavioural problems were evenly distributed across the genotype subgroups. Our findings support the notion that mutations towards the 3'end of *dystrophin*, which disrupt not only the long products, but also in turn the short brain-expressed isoforms Dp140 and Dp71, have a more devastating effect on the neurocognitive phenotype (table

14).[66, 67] Furthermore, the long dystrophin products Dp427 also must play a significant role in the association of CNS comorbidities. Proximal mutations are sufficient to cause cognitive and neurobehavioral problems, hence Dp427 is also important for the neurodevelopment of the brain. This should not be surprising considering that Dp472 is largely expressed in the cortex, hippocampus and cerebellum, localizing to neuronal GABAergic synapses. In the mdx mouse, lack of Dp427 is associated with reduced receptor clustering, impairing specific amygdala GABAergic transmission and enhancing defensive behaviour in response to danger.[219] In contrast, Dp140 localizes in glial cells and Dp71 is expressed in the perivascular astrocytes and Dp71 is required for the anchoring of aquaporin-4. Loss of Dp71 is associated with reduced levels of aquaporin-4, therefore altering trans-membrane water permeability.[220, 221]

We could speculate that, when in addition to Dp472 also the short dystrophin products are disrupted, a "compensatory/protective" mechanism produced by these abundantly CNS-expressed isoforms may be lost, increasing the risk of associated neuropsychiatric disorders and accounting for the more devastating profile observed in boys with Dp71 mutations.

Yet much remains unclear of the effects of dystrophin disruption in the CNS, and how different isoforms contribute to pathology. Structural abnormalities in the brain have been recently described: changes in the cerebral grey matter volume and white matter microstructure in boys with DMD have been described using quantitative MRI.[222] Previous studies using MR spectroscopy have indicated altered phosphorous metabolite ratios in the cerebral cortex of 19 DMD boys.[223] In the *mdx* animal model, disrupted cerebral diffusivity was demonstrated associated to lack of dystrophin.[224]

Finally, novel pharmaco-gene therapies aiming at restoring dystrophin expression and delaying the course of the disease progression are currently in different phases of experimentation. Some of these compounds, such as the tricyclo-DNA antisense molecules, are also capable of crossing the blood brain barrier and restoring dystrophin expression through exon skipping in the CNS. This approach demonstrated clinical benefit and behavioural amelioration in the *mdx* mouse model, after receiving systemic antisense administration, indicating that at least some aspects of the dystrophin-associated neurodevelopmental syndrome might be reversible upon brain dystrophin restoration.[225]

Our study has some limitations: although a large cohort of patients was recruited, the distribution between genotype sub-groups was not equal, this was adjusted for in post-hoc analysis; we did not have reports from the school to match observations from parents, with the exception of 41 Conners from teachers; parents' reported measures may have overestimated neurodevelopmental problems, therefore a more in depth assessment is required for diagnostic purposes; both the 3Di and the SCDC were used for the first time outside the UK, therefore normative data does not exist for Italian and Belgian populations; finally, we have speculated that that our findings are secondary to dystrophin deficiency in the CNS, however such speculation requires further studies elucidating the nature of this relationship.

Despite its limitation, this study fuels the growing interest in unravelling the yet unclear role that the disruption of different dystrophin isoforms may play in the brain development and function, and provides a baseline to evaluate if these events can be reversed and to what degree, with emerging dystrophin- restoring therapies.

CHAPTER 5: MANIFESTATIONS OF DYSTROPHIN DISRUPTION IN THE DMD RETINA

The work contained in this chapter has given rise to the following publication:
Ricotti V, Jägle H, Theodorou M, Moore AT, Muntoni F, Thompson DA. *Ocular and neurodevelopmental features of Duchenne muscular dystrophy a signature of dystrophin function in the central nervous system*. Eur J Hum Genet. 2015 Jun 17. [Epub ahead of print] PMID: 26081639

My contribution to this work consisted of the following:
I oversaw the design of the study. I led recruitment of patients into this study and contributed to data collection under the supervision of Dorothy Thompson. In collaboration with Dorothy Thompson I analysed the data. I wrote the first draft of the manuscript, and contributed to the revision of the manuscript.

The human retina

The human retina is a complex layered structure with a large diversity of cells and an intricate circuit and connections, which work in parallel and combination to produce a composite visual output.

The retinal layer encompasses 5 major neuronal cell classes (figure 18) : 1) Rods and cone photoreceptors, 2) Horizontal cells; 3)Bipolar cells; 4) Amacrine cells; 5) Ganglion cells. In addition Müller glial cells provide metabolic and homeostatic support. Photoreceptors are classified into rods and cones; with rods having exquisite sensitivity to light, thus responsible for dim-light vision; and cones being much less sensitive than rods, but exhibiting much faster response

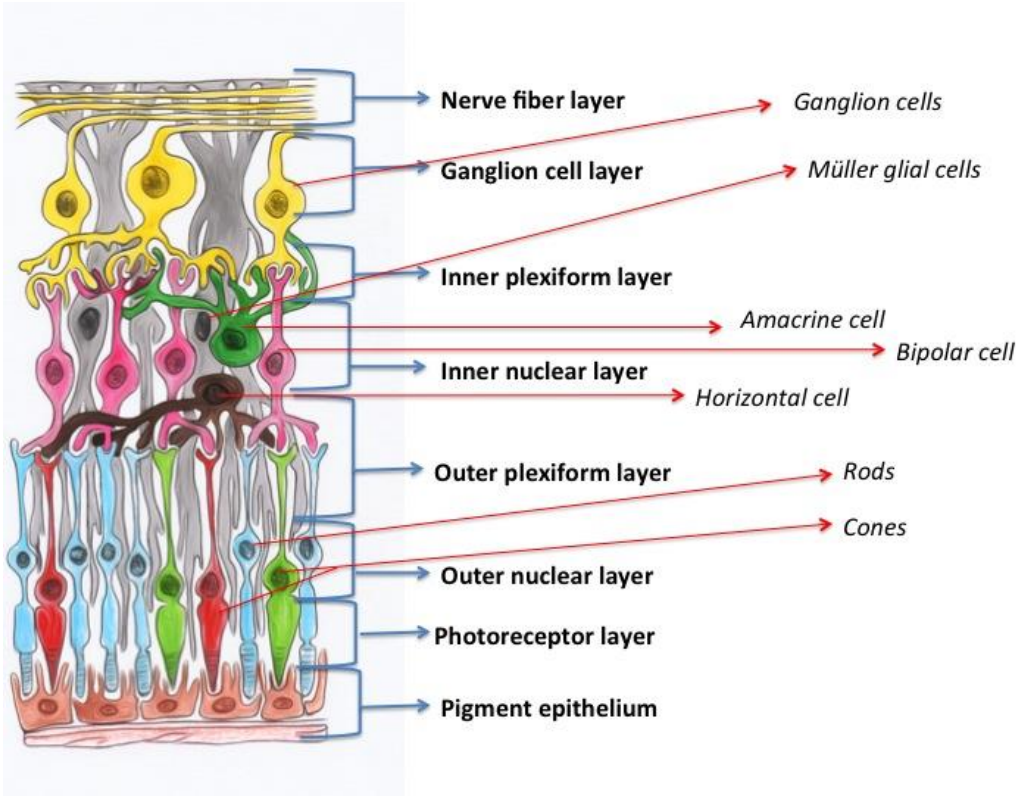
during photo transduction, therefore responsible for bright-light, high acuity colour vision. Moreover, each cone photoreceptor type is most sensitive to a specific wavelength of light.

Coding of visual information happens with conversion of light energy to membrane potential changes in these photoreceptors, which by the use of glutamate alters neurotransmitter release. Cones and rods synapse onto second order glutamatergic bipolar cells at the outer plexiform layer (OPL). Bipolar cells are either rod or cone connected, and are further divided into 2 functional groups: those that depolarise (i.e. ON) and those that hyperpolarise (i.e. OFF) in response to augmentation in light intensity. Bipolar cells in contact with rods are exclusively ON, whereas cone bipolar cells can be of either type. Moreover, horizontal cells modulate such synaptic transmission: they possess dendritic connections between cones, and in addition have an axon with extensive terminal arborisation with rods. Despite the modulating effect of horizontal cells, most of the signal compression and information processing is done by the amacrine cells in the inner plexiform layer (IPL). Cone bipolar cells synapse with retinal ganglion cells and amacrine cells forming the IPL. Amacrine cells receive signals from a very large number of connecting bipolar/ horizontal cells and directly modulate the excitation of retinal ganglion cells through either direct or indirect inhibition pathways, largely mediated by GABA and glycine. Within the IPL, synaptic connections are further organized in two distinct layers. The inner lamina of the IPL comprises synapses between ON-bipolar cells and retinal ganglion cells and amacrine cells, whereas the outer lamina contains synaptic connections of OFF-bipolar cells.[226]

The last cells in this complex circuit are the ganglion cells, which come in several varieties and sizes. Ganglion cells are the sole output neurons of the retina, projecting their axons to higher visual centres.[227, 228]

Finally, the Müller glial cells, which span across the entire thickness of the neuronal retina, are essential for supporting neuronal cell metabolism. However, a recent study has shown that the enhancement of signal by Müller glial cells greatly contributes to increase the clarity of vision by channelling green and red colours, mostly needed for day vision to the cones and scattering blue light, mostly needed for night vision to the neighbouring rods, therefore further optimising day and night vision.[229]

Figure 18. Retinal layers and cells



(Graphic by Gabriella Codastefano)

Figure 19. The electroretinogram

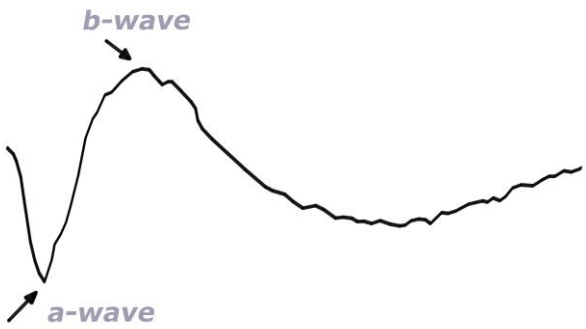


Figure 19. The human electroretinogram: the two components that are most often measured are the a- and b-waves. The a-wave (negative) =physiological response of photoreceptors in the outer retina; b-wave (positive) = inner layers of the retina

The electroretinogram

The electroretinogram (ERG) offers a way of measuring combined electrical responses of the various retinal cell classes. The basic method of recording the electrical response (full-field ERG) is by stimulating the eye with a bright light source. The flash of light elicits a biphasic waveform recordable at the cornea. The two components that are most often measured are the a- and b-waves (figure 19). The a-wave is the first large negative component, and reflects the physiological response of photoreceptors in the outer retina. This is followed by the b-wave which is corneal positive and usually larger in amplitude, and reflects the response of the inner layers of the retina (including the ON bipolar cells) and the Müller glial cells. The 2 principal measures of the waves recorded are the amplitude and the time. Furthermore the wavelets, which occur on the rising phase of the b-wave, are known as oscillatory potentials (OPs), and are thought to reflect activity in amacrine cells.[226, 227]

By manipulating adaptation level and background illumination, flash intensity, color of the flash and rate of stimulation, rod and cone activity can be significantly isolated and recorded. [226]

Dystrophin and the retina

As discussed in *Chapter* 4, the *DMD* gene is a very large gene with 79 exons and tightly regulated internal promoters, which make a range of protein isoforms, identical at the C-terminus, but with unique N-termini. These include 3 full-length dystrophin isoforms (Dp427) and the shorter products Dp260, Dp140, Dp116 and Dp71. A major function of dystrophin in muscle is to protect muscle fibres against the mechanical forces of contraction, thus its absence renders muscle fibres susceptible to stretch-induced damage and necrosis.[188] Dystrophin has also been assigned a signalling role; while some of these activities are relevant for skeletal muscle function (e.g. modulation of adrenergic tone by nNOS)[26], others are essential for dystrophin function in organs such as brain and retina.[189, 190] Furthermore, different dystrophin isoforms are localised in distinct regions of the CNS and the retina (figure 17, chapter 4). As discussed in chapter four, Dp140 and Dp71 are largely expressed in the brain, and the lack of both these isoforms is well recognised to be associated to higher incidence of cognitive impairment and neurodevelopmental disorders in patients with mutations downstream exon 45.[67, 68, 72] All dystrophin isoforms, with the exception of Dp116, are expressed highly in the retina (figure 20). Dp427, Dp260 and Dp140 are located in photoreceptor terminals, while Dp71 is expressed in Muller glia cells.[230-235] Recently Dp427, Dp260 and Dp140 expression have been identified in inner retinal layer neurons as well; Dp427 was expressed proportionately more at cone than rod synapses, in bipolar cells and some amacrine cells.[233] These recent findings suggest that Dp260 and Dp71 are not redundant protein products in the retina. Their location in different sites within the retina suggests that their contribution to retinal electrophysiology is indeed distinct.[235]

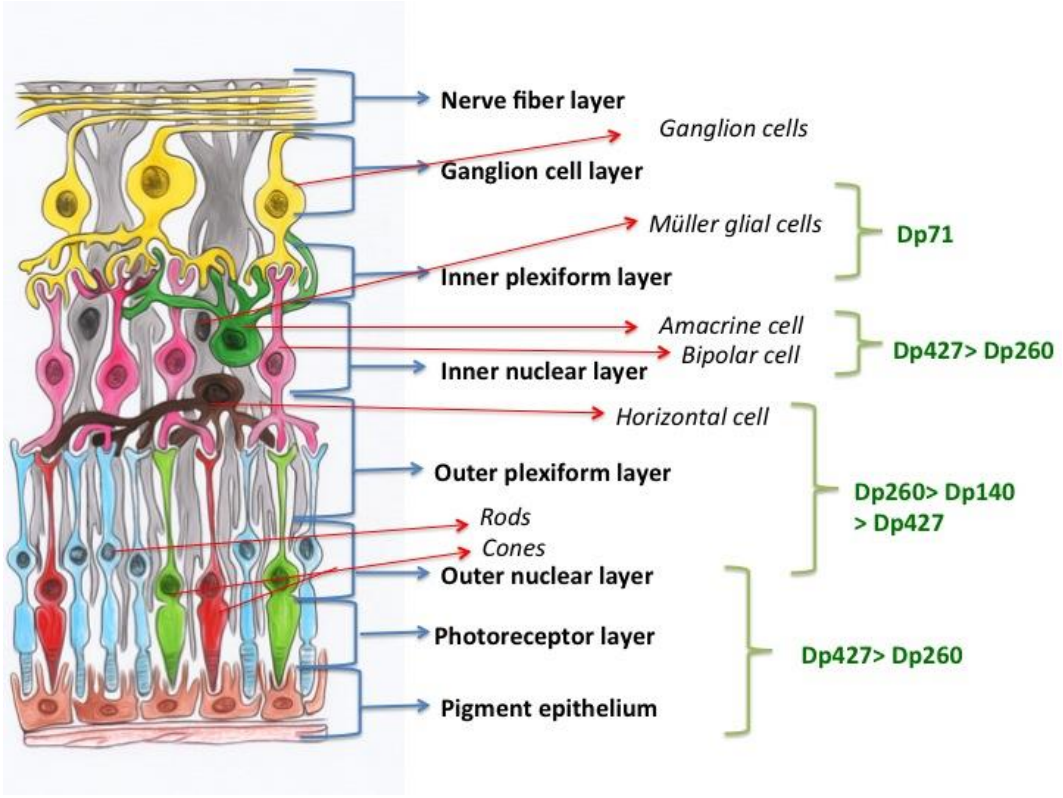
It is well recognised in the literature that absence of dystrophin from the retina gives disturbances in retinal electrophysiology.[236] More specifically, mutations downstream of exon 30, which affect Dp260 expression, are associated with an abnormal scotopic ERG waveform, as demonstrated both in mouse models[190, 237-239] and in individuals with Duchenne/Becker Muscular Dystrophy.[191, 240]

Pillers *et al*. reported that DMD subjects with normal ERGs had mutations predominantly at the 5′ end of the *DMD* gene (n=7/14), whilst all subjects studied with mutation predicted to disrupt production of Dp260 (n=50) showed marked reduction of b-wave amplitude.[191]

More recently Barboni *et al.* tested 19 DMD patients and 7 heterozygous DMD carriers with deletions, duplications, and point mutations against 19 age-matched controls.[240] The study showed a reduction in scotopic positive peaks of the ERGs across the genotype in DMD subjects; however more severe changes were found in patients with mutations downstream exon 30, hence affecting Dp260. At photopic level alterations were of similar extent both in patients mutated downstream and upstream exon 30. Furthermore, some alterations were also observed in DMD carriers but these findings were not significant when compared to controls. Finally contrast sensitivity was also tested and found to be significantly lower in DMD subjects.[240]

Red-green colour vision impairment was also reported in DMD and in association with Dp260 disruption. [241] Costa *et al.* evaluated colour vision in 44 DMD patients against a control group of 70 age-matched healthy males: over half of patients with mutations downstream exon 30 had red-green colour vision impairment as measured with the Cambridge Colour Test, while subjects with mutations upstream exon 30 had normal colour vision. [241]

Figure 20. Schematic representation of expression of Dystrophin in the retinal layers



(Graphic by Gabriella Codastefano)

A study on ocular and neurodevelopmental features of Duchenne muscular dystrophy: a signature of dystrophin function in the central nervous system

In light of the recent retinal expression studies, this study sought to explore the clinical ERG as a potential biomarker of CNS dystrophin expression associated with different DMD genotypes and recorded ERGs to a wide range of flash strengths in a cohort of individuals with a range of mutations across the *DMD* gene. A subsidiary, but important, aim was to assess in this population whether a validated non-invasive paediatric ERG protocol would provide results comparable to those from a more demanding international standard method.[242] Finally, we explored a potential relation between ERG and the neuropsychiatric profile using validated psychometric inventories.

Methods

Participants

Sixteen DMD boys attending the neuromuscular outpatient Department at Great Ormond Street Hospital (GOSH) were recruited according to their genotype, ensuring balanced distribution of mutations downstream and upstream of exon 30 (table 17), but also tolerability of the assessment. The diagnosis was confirmed by finding an out-of-frame-frame mutation in the *DMD* gene, using Multiplex Ligation-dependent Probe Amplification (MLPA). Where DMD deletions or duplications were not identified, all 79 exons and the adjacent introns were analyzed through PCR amplification and direct sequencing to

screen for point mutations. Mutations were classified according to the Leiden Muscular Dystrophy database.[123]

Seven boys had mutations involving exons 3-13 (affecting expression of Dp427 isoforms); 7 boys had mutations involving exons 44-57 (disrupting Dp427, and progressively also Dp260, Dp140 and Dp116); 2 boys had mutations downstream exon 63 (disrupting all dystrophin isoforms, including the shortest Dp71). All patients were fully cooperative and able complete the examination, with the exception of the 2 boys with Dp71 mutation who had a profound intellectual disability, as expected for this genotype,[67] and could only complete the paediatric protocol. The mean age was 11 years (range 4-15). All the boys were on prednisolone given at 0.5- 0.75 mg/kg/day daily or intermittently (10 days on:10 days off).[32, 33, 243]

Table 17. General Characteristics of DMD subjects in this study

ID	Age at assessment (years)	Mutation	Dystrophin isoforms predicted to be affected*	Neuro-behavioural problems	GAI
1	7	Deletion exon 3-7	Dp427	SCDC = 7 Internalising behavioural problems	98
2	10	Deletion exons 3-7	Dp427	SCDC = 2 No	102
3	15	Deletion exons 5-7	Dp427	SCDC = 7 Attention deficit and anxiety	90
4	11	Deletion exons 8-13	Dp427	SCDC = 4 No	110
5	10	Deletion exons 3-11	Dp427	SCDC = 4 No	119
6	10	Deletion exons 8-13	Dp427	SCDC =16 Inattention, internalising behavioural problems and ASD	83
7	8	Duplication exon 2	Dp427	SCDC = 3 No	90
8	13	Duplication exons 56-57	Dp427, Dp260, Dp140, Dp116	SCDC =10 Internalising behavioural problems and anxiety	119
9	8	Duplication exons 56-57	Dp427, Dp260, Dp140, Dp116	SCDC = 9 Attention deficit	96
10	8	Deletion exons 51-54	Dp427, Dp260, Dp140	SCDC = 9 Internalising behavioural problems	119
11	12	Deletion exons 48-50	Dp 427, Dp 260, Dp 140	SCDC = 0 No	116
12	11	Deletion exon 44	Dp 427, Dp 260, Dp 140	SCDC = 2 No	99
13	14	Deletion exons 51	Dp 427, Dp 260, Dp 140	SCDC =1 No	106

14	10	Deletion exons 49-54	Dp 427, Dp 260, Dp 140	SCDC =1 Attention deficit	90
15	11	Deletion exon 70	Dp 427, Dp260, Dp 140, Dp116, Dp 71	SCDC =17 ASD	N/A*
16	4	Nonsense mutation exon 70	Dp 427, Dp 260, Dp 140, Dp 116, Dp 71	SCDC =16 ASD	N/A*

Table 17: General Characteristics of DMD subjects in this study

A total score on the *Social and Communication Disorder Checklist* (SCDC) ≥ 8 is suggestive of emotional socio-communicative behavioural problems; a total SCDC score of >15 is invariably associated with a diagnosis of Autistic Spectrum Disorder (ASD). IQ is expressed as a General Ability Index (GAI) score and calculated from a truncated version of the WISC-IV. Behavioural problems are reported as meeting the threshold scores in the Parents' Conners 3, Child Behavioural Check List and the short version of the Developmental Diagnostic and Dimensional Interview (3Di-sv).

* Severe speech delay/non-verbal

Experimental protocol

All patients underwent eye examinations: visual acuity, Ishihara colour vision assessment, intra-ocular pressure (IOP) measurement, fundoscopy and electrophysiology examination of the retina using Electroretinograms (ERG). ERGs were recorded both to international standards (ISCEV)[244] and with a modified paediatric protocol developed and validated at GOSH. [245, 246] The paediatric GOSH protocol does not require lengthy dark adaptation, dilation, or corneal electrodes and therefore has greater applicability in uncooperative children. ERGs are recorded from peri-orbital skin electrodes in response to flash stimuli delivered by a hand held Grass strobe under photopic and scotopic conditions (figure 21). This method elicits ERGs which are of smaller amplitude, but physiologically comparable to the diagnostic International Society of Clinical Vision Society (ISCEV) standard protocol.

The range of flash luminance presented in the dark and light were extended to more specifically probe slow and fast signalling pathways in the retina. Images of retinal macula structure and retinal nerve fibre layer thickness were taken when possible with a high resolution Fourier optical coherence tomographer (OCT) (Heidelberg Spectralis) device. The intellectual quotient (IQ), where possible, was assessed with a truncated version of the Wechsler Intellectual Scales for Children-Fourth Edition (WISC-IV)[198], which included Vocabulary, Similarities, Matrix Reasoning, Block Design, Digit Span and Letter-Number Sequencing. The IQ was expressed as a General Ability Index (GAI) score[247]. The boys' families were asked to complete the Social and Communication Disorders Checklist (SCDC).[196] The SCDC is a validated 12-item measure for neurodevelopmental disorders. A total score of ≥8 is indicative of neurodevelopmental disturbances, especially in the social communication

domain, whilst scores >15 are strongly suggestive of autistic spectrum disorders (ASD).[197] Families who reported behavioural problems were asked to participate in further assessments including: the Developmental, Diagnostic and Dimensional Interview short version (3Di-sv) to assess Autistic Spectrum Disorder (ASD)[204], Conners' Parent Rating Scale (Conners 3)[205] to assess Attention Deficit Hyperactivity Disorder (ADHD); and the Child and Behavioural Check List (CBCL)[205] to assess internalising and externalising emotional problems.

Figure 21. Patient assessment using the modified paediatric GOSH ERG protocol

(Courtesy of the Academic Department of Ophthalmology, Great Ormond Street Hospital for Children, London, UK)

Statistics

The amplitudes and peak times of ERG a- and b-waves from the patients were compared with clinical laboratory age-matched normative data for the ISCEV and GOSH protocols expressed as 5th and 95th centiles. The ratios of a:b wave amplitudes to scotopic and photopic standard flash stimuli obtained with the GOSH and ISCEV recording techniques were compared using a Pearson correlation. A one-way ANOVA comparing ERGs and SCDC scores between the genotype groups was performed using GraphPad Prism.[248] P-values <0.05 were considered significant.

Results

The ERG waveform has a negative a-wave due to the photoreceptors hyperpolarizing in response to light followed by a positive b-wave that depends upon signalling between photoreceptors and bipolar cells depolarizing the bipolar cell. Across all genotypes cone photopic b-wave amplitudes were subnormal, especially to high flash luminances, whilst photopic a-wave amplitudes were ≥5th centile, indicating normal cone photoreceptor function (figure 22). In contrast scotopic a-wave amplitudes were subnormal falling below the 5th centile in 6/16 (40%) of tested patients (figure 23, bottom graph). Individual scotopic ERG a- and b-wave amplitude are plotted against normative data (figure 23). Boys with mutations downstream of exon 30, affecting both Dp427 and Dp260 (individuals 8-16, table 1), showed subnormal scotopic b-wave amplitudes to all flash strengths. As the amplitude of the preceding a-wave was larger than the b-wave the a:b amplitude ratios were >1 and the resulting waveform is called electronegative (figure 22).

Individuals with mutations affecting only Dp427 (individuals 1-7, table 17) showed milder scotopic b-wave amplitude reduction, with a:b ratios of 1 (normal a:b ~1:2) (figure 22). The only exception was individual 6, who had a deletion of exons 8-13, yet showed a similar scotopic ERG profile to boys with mutations affecting Dp260.

Boys with mutations affecting all protein products, including Dp71 (individuals 15-16, table 17), showed the most profoundly electronegative scotopic ERGs (figure 24).

When comparing photopic and scotopic mean a:b ratios we found a significant difference between mutations upstream and downstream of exon 30 ($p<0.001$). Furthermore, the a:b ratio data obtained by the ISCEV corneal electrode protocol were comparable with the GOSH skin electrode protocol ($r=0.87$, $p=0.01$) (Figure 25).

The scotopic and photopic ERGs were filtered to reveal 4 oscillatory potentials. Boys with Dp427 mutations had a small cone OP2, but normal rod OPs. Those with mutations downstream exon 30 had an absent rod OP2, but surprisingly cone OPs in some were normal (figure 26).

OCT axial sections performed randomly in 6 individuals, showed normal retinal lamination and macular profiles. The retinal nerve fibre layer thicknesses were within normal limits across genotypes (figure 27).

Figure 22. ISCEV ERG trace examples.

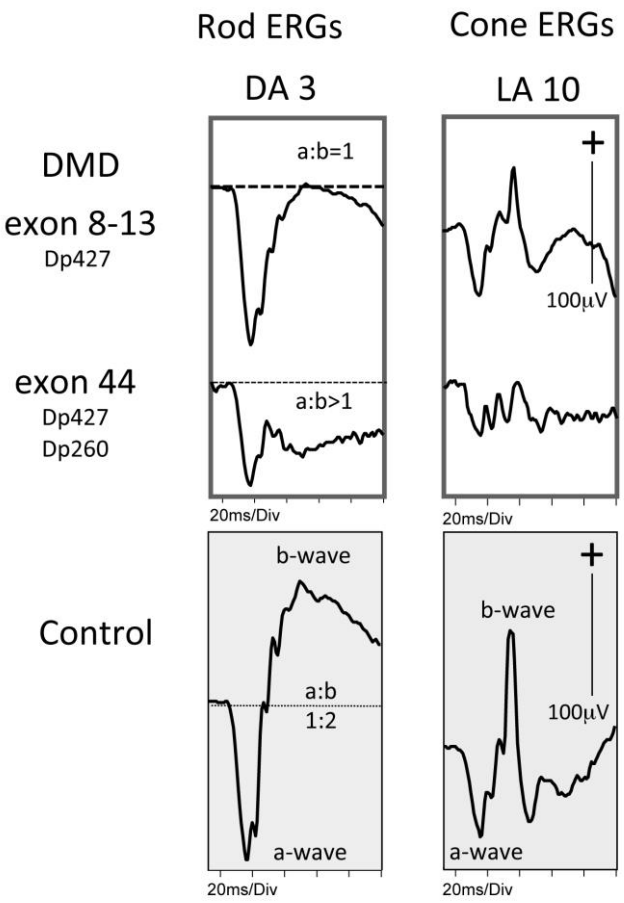


Figure 22. Examples of rod and cone ISCEV full field ERGs from patients with mutations at 5′ and 3′ end of the gene are compared with normal waveforms, in which the a-wave is smaller than the b-wave and the a:b ratio is 1:2. The upper traces from a boy with a mutation affecting Dp427 alone (i.e. exons 8-13) show a DA 3 rod ERG in which the b-wave is reduced to the same size as the a-wave giving an a:b ratio of 1. Below, an individual with a mutation affecting Dp427 and Dp260 (i.e. exon 44) with a DA 3 rod ERG b-wave that is smaller than the a-wave giving an electronegative ERG. The cone ERG LA 10 waveforms show a similar pattern with normal a-waves, but small b-wave amplitudes altering the overall ERG waveform.

Figure 23. ISCEV scotopic dark-adapted ERG measurements

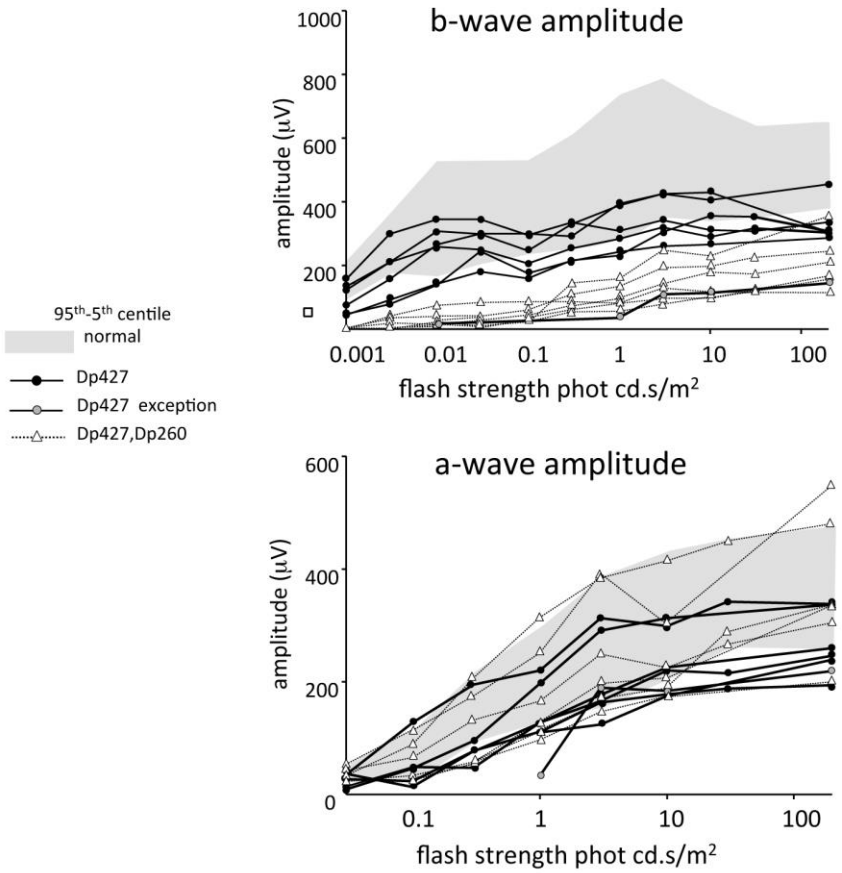


Figure 23: ISCEV scotopic dark-adapted ERG measurements

Top graph: Dark-adapted b-wave amplitude is plotted against flash strength. It shows a dichotomy of Dp427 (filled symbols) and Dp260 (open symbols) response, accentuating the subnormal amplitude when expression of Dp260 is compromised. The b-wave time to peak is also distinctive, falling below normal range i.e. shorter time to peak, to high increasing flash strength when only the Dp427 expression is affected.

Lower graph: a-wave amplitude is plotted against flash strength. Dp427 (filled symbols), are more often seen below the 5th centile at high flash strengths than Dp260 (open symbols), but the time to peak for both fall within normal range. The exception, shown as a grey filled symbol, is from a patient with deletion of exons 8-13.

Figure 24. Skin ERG trace examples

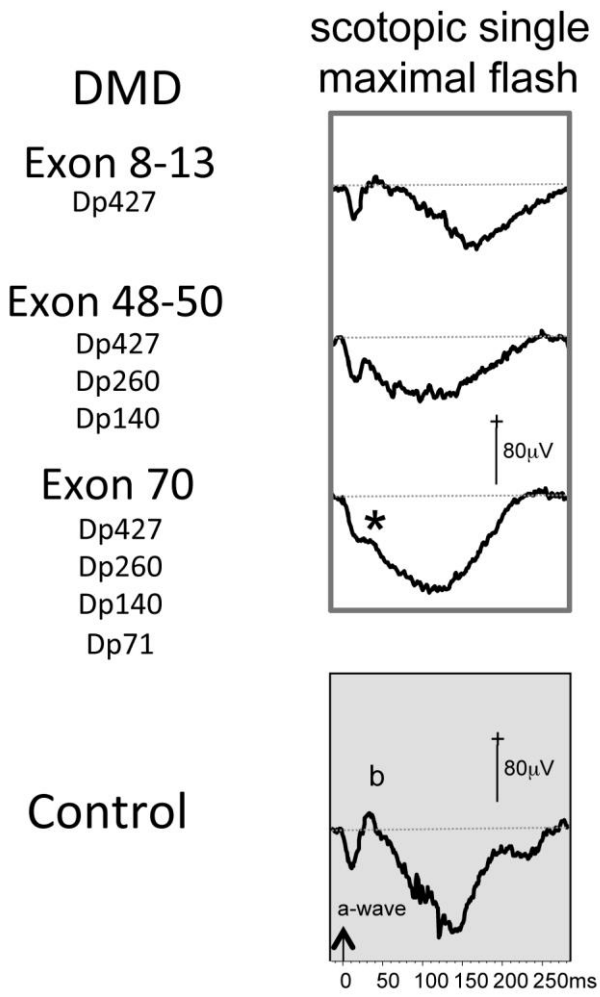


Figure 24. Skin ERG trace examples. Examples of skin ERGs are shown to illustrate responses from individuals with mutations affecting each isoform. The absent b-wave, (marked with *), in association with a mutation in exon 70 results in a profoundly electronegative ERG waveform. The trace time base is extended to 250ms to show that eventually the polarity of the ERG recovers and the trace re-joins the baseline.

Figure 25. Scotopic and photopic a:b ratios ISCEV standardised protocol and GOSH protocol

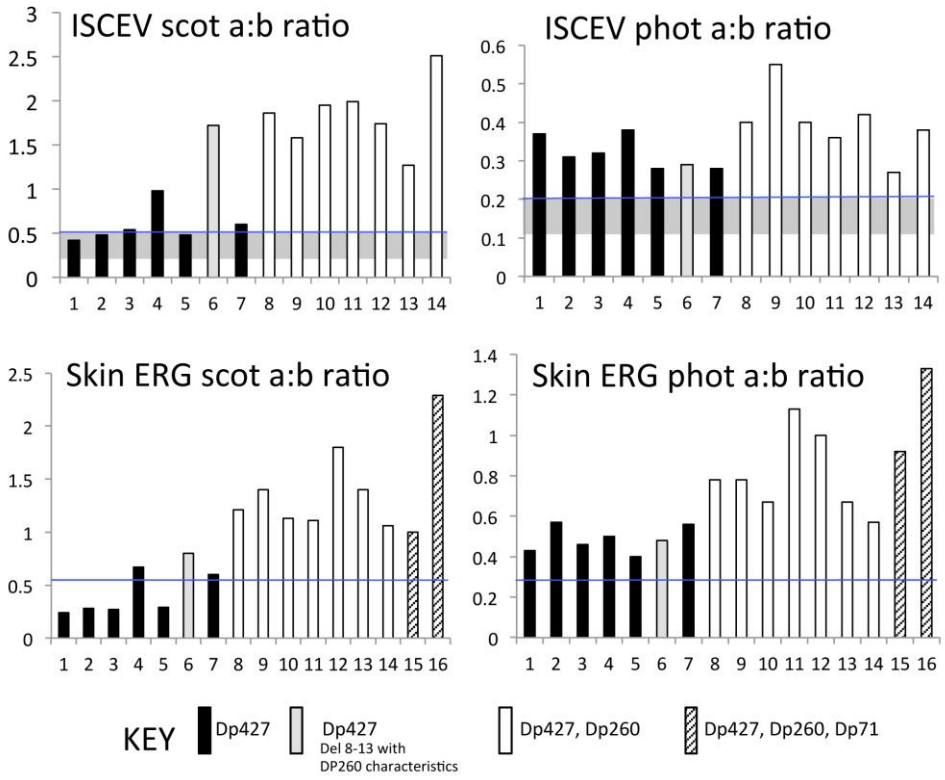


Figure 25: Scotopic and photopic a:b ratios ISCEV standardised protocol (top) and skin GOSH protocol (bottom). These bar charts show the ratio of a:b-wave amplitudes for each patient. These are arranged according to the isoform affected. The horizontal lines indicate the normal reference ratio. Data falling above the line indicates a low b-wave amplitude and abnormal a:b ratio. The top left graph shows the strong association of Dp260 (open bars) with electronegative DA ERGs as the a:b ratio is greater than 1. The top right graph abnormal cone ERGs a:b ratios occur mildly across all mutations. Patients with mutations affecting Dp71 managed skin ERGs (GOSH protocol) only depicted on the lower graphs, not requiring formal dark adaptation; these are electronegative for both photopic and scotopic conditions. The exceptional case (individual 6) is highlighted next to the Dp260 patients: although his mutation is predicted to affect only Dp427, the ERGs suggest Dp260 expression is also affected. Importantly, the same profile of a:b ratio with genotype is seen for the skin

GOSH ERG data as ISCEV ERG data (r= 0.87, p= 0.01) i.e. ratios fall above the line for the same patients.

Figure 26. Oscillatory potentials

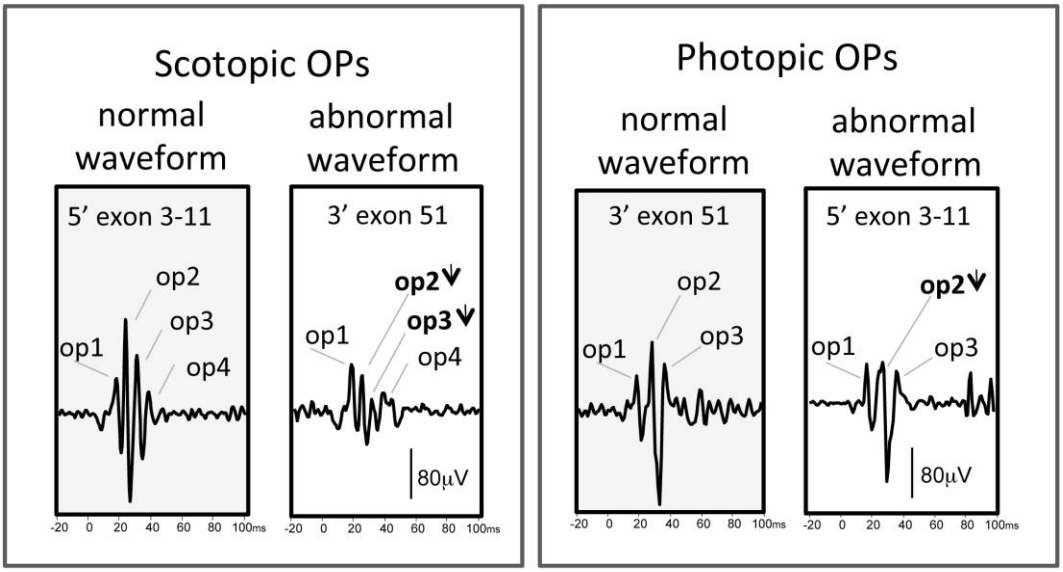


Figure 26. The oscillatory potentials (OPs) are high frequency wavelets revealed by filtering the ERG trace between 100-300Hz. These are numbered for identification, though loss includes the possibility of OP delay and consequent merger with other OPs. The photopic OP2 in the patient with a mutation at the 5' end of the gene is subnormal, but the scotopic OPs are normal. Scotopically the greater effect of Dp260 is seen and these scotopic OP2 and OP3 are subnormal, yet unexpectedly this patient has normal photopic OPs.

Figure 27. Retinal structure

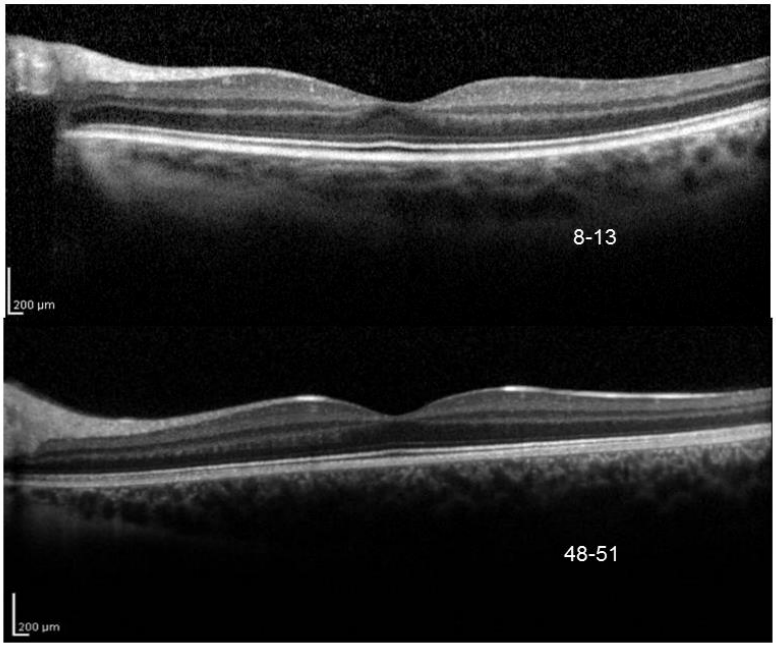


Figure 27: Optical coherence tomography (OCT) show normal retinal structure with resolution of 7-10 microns).
(Courtesy of Dorothy Ann Thompson Clinical and Academic Department of Ophthalmology, Great Ormond Street Hospital for Children, London, UK)

Clinically, all boys had normal fundi and normal visual acuity. One boy with a nonsense mutation in exon 70 had a red/green colour vision defect. IOPs were mildly elevated in two boys (22-24mmHg).

Explorative relation between ERG findings, behavioural problems and intellectual disability

All the subjects recruited in this study were cooperative and with normal range GAI, with the exception of the 2 boys with Dp71 mutations, who had a profound speech delay. Nine of the 16 boys had neurobehavioural problems: 5/6 boys who scored high in the SCDC had mutations downstream of exon 30 and scored above the threshold for ADHD, ASD and emotional problems (table 17). Two of seven boys with mutations upstream of exon 30, whose families reported mild behavioural disturbances, had scores above the threshold for inattention and anxiety (table 17). The two boys with mutations downstream of exon 63 and profound speech delay, scored in the ASD range according to both to the SCDC and the 3Di-sv. These two boys only managed the GOSH ERG skin protocol. Interestingly, the same boy (individual 6), with 5'-end mutation, but with a Dp260, 3'-end mutation type of ERG profile also presented with a neurocognitive profile (i.e. inattention, emotional problems and ASD) comparable to that of children with 3'-end mutations. In this subject we therefore suspected a second mutation in addition to the deletion of exons 8-13 identified by MLPA and we carried out a full dystrophin gene sequencing, which however did not reveal other mutations.

Exploring a possible relationship between ERG and neurobehavioural outcomes we compared the SCDC scores as an overall index of neurodevelopmental impairment with scotopic a:b ratios. Although our analysis did not meet statistical significance ($p=0.1$), we observed that boys with electronegative scotopic ERGs to score more likely in the abnormal range for the SCDC (6 out of 10), whilst none of the six boys with electropositive scotopic ERGs had abnormal scores (figure 28).

Figure 28. Social and Communication Disorders Checklist (SCDC) score according to ERG findings

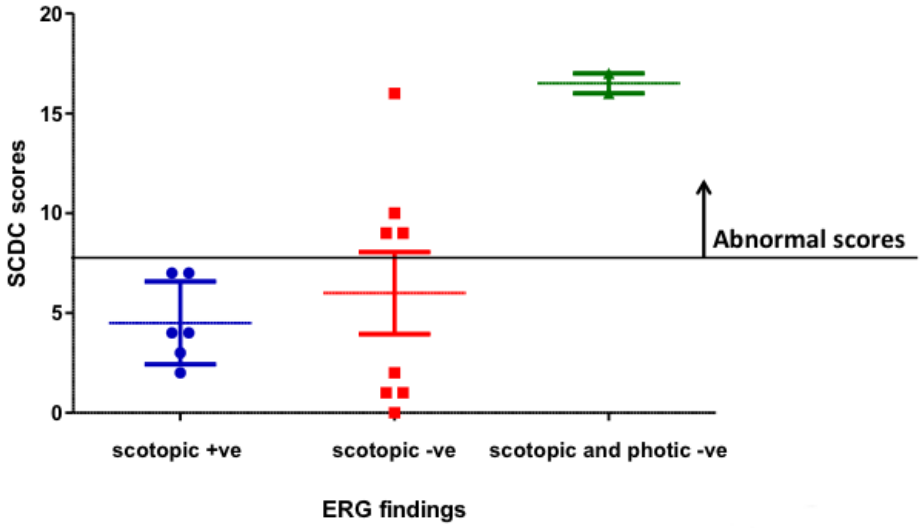


Figure 28. ANOVA comparing SCDC scores in electronegative and electropositive scotopic ERG groups (Graph pad Prism)[248]. SCDC scores above 8 are abnormal and highly indicative of neurodevelopmental disorders. Boys with electronegative ERGs were more likely to have abnormal scores (p=0.01).
P value< 0.05 was considered significant.

Discussion

In collaboration and with the support of the ophthalmology department at GOSH, I studied the retinal physiology and neurodevelopmental profile of 16 DMD boys, with a range of mutations around a 'pivot' region of interest at exon 30, which enabled us to segregate CNS/retinal expression of shorter isoforms Dp260, Dp140, and Dp71 from the full-length dystrophin isoforms Dp427 which are predominantly expressed in muscle[18]. We examined the retina functionally with the ERG, and structurally with OCT imaging. We found ERG changes corresponding with the recently reported differential expression of Dp isoforms between retinal sub-networks in animal models, with Dp427 allied more with cone pathways and Dp260 with rods.[233]

We now recognise that ERG b-wave amplitude mainly depends upon depolarisation of on-bipolar cells[249], rather than potassium channels on the Muller glial cells,[250] which are altered in the Dp71 null mouse models of DMD. This explains why ERGs reported in DMD patients differ from some previously predicted DMD mouse knockout models.[231, 237]

In this study DMD patients selectively lacking expression of Dp427 isoforms showed a preponderance of changes in cone b-waves indicating a disruption of signalling between normally functioning cone photoreceptors and cone on-bipolar cells. In contrast, rod a-wave amplitude was subnormal, indicating altered rod photoreceptor function in 5/7 of patients with mutations affecting Dp427 alone. This has not been reported before and may be due to the brighter flash used in our study. Expression of the Dp260 isoform, between rod photoreceptors and rod on-bipolar cells, was necessary for slow rod pathway function. Altered expression of Dp260 caused subnormal b-wave amplitude and hence electronegative scotopic ERG waveforms. This characteristic signature was

exacerbated if Dp71 was additionally compromised, as shown in figure 24. In our study we showed that, indeed, mutations affecting Dp71 have the most profound and uniquely electronegative scotopic ERG (figure 24), alongside showing most severe cognitive-neuropsychiatric profile disturbances.

This study highlighted only one exception in such patterns: a boy with a mutation of exons 8-13 (i.e. affecting Dp427 alone) showed both an electronegative scotopic ERG, severe speech delay and a neuropsychiatric profile in the autistic spectrum domain. Additional *DMD* mutations were not identified; the combined ERG and neurodevelopmental phenotype of this child remain unexplained.

Retinal signalling depends upon the structural alignment of proteins at the synapses. Dystrophin is considered a developmental determinant of structural apposition of photoreceptors with bipolar cells.[251] It was anticipated that patients with DMD might show altered patterns of retinal lamination, but high-resolution spectral domain OCT axial images of the in vivo retina did not show any irregularity of the outer plexiform lamina nor abnormal lamina thicknesses in a comparison with normal.

As retinal lamination is normal, the observed ERG changes are more likely due to altered synaptic transmission and membrane polarisation. This implies a role for dystrophin isoforms in retinal signalling. Our novel evidence of isoform-specific loss of rod and cone oscillatory potentials supports this contention as OPs are generated by spiking amacrine and ganglion cells.

In relation to the neuropsychiatric profile, an important consideration is required: in order to facilitate electrophysiological assessments, the recruitment was biased towards DMD boys with good understanding and co-operation. With the exception of the two Dp71 boys, who had profound delay, the GAI scores

were within normal ranges. Nevertheless neurobehavioural problems were observed in nine boys and including ADHD, ASD and emotional problems. Although three boys with Dp427 mutations presented with some behavioural problems, overall boys with mutations progressively affecting the shorter dystrophin isoforms were more commonly and more severely affected. This is not surprising, as the full length isoforms are also expressed in the neuro-cortex, hippocampus and cerebellum. However, the shorter isoforms are most abundantly expressed in the brain, with the pivot region of interest for the brain in exon 45. Indeed, Dp140 has been amply localized in glial cells,[252] and disruption of this isoform in mutations affecting exons 45-62, has been implicated with cognitive impairment, higher incidence of ADHD and delayed milestones.[12, 68, 72] Mutations affecting Dp71, a brain isoform implicated in trans-membrane permeability,[220] although rare are recognized in association with severe cognitive impairment.[68, 213] The two boys with Dp71 mutations, indeed had the most severe profile with ASD and profound speech delay. Our findings, in line with previous reports in the literature, suggest a possible cumulative effect on neurodevelopment caused by the progressive loss of the shorter dystrophin isoforms, with mutations towards the 3'-end of *DMD* being associated with the most devastating outcomes.

In our cohort, of clinical importance, we observed that scotopic electronegative ERGs were more likely to be associated with neurodevelopmental disorders. However, the spectrum of neuropsychiatric morbidities in dystrophinopathies is vast, complex and multifactorial; our sample population was relatively small; and our observations were based on validated questionnaires/interviews rather than extensive neuropsychological assessments. Therefore, an association

between retinal electrophysiology and neurodevelopmental disturbances needs further exploration in much larger cohorts.

Experimental treatments with antisense-mediated exon skipping or drugs that allow read-through of nonsense mutations are currently being tested in human clinical trials, demonstrating successful partial restoration of dystrophin expression in muscle.[78, 79] A new class of therapeutic agents, have recently shown to be able cross the blood brain barrier in animal models and to restore dystrophin also in the CNS,[225] therefore there is both the need to better understand the DMD CNS changes and to identify the treatment response with quantifiable biomarkers.

This study shows that retinal electrophysiology is a tool sensitive to dystrophin protein absence in the CNS; it provides an individual patient pre-treatment retinal response profile as a signature of protein disruption. In absence of structural defects in the eye of DMD boys, we can speculate that the electrophysiological signature may change towards normal as protein is restored also in the CNS, however this model requires further exploration in the retina, while it was demonstrated in the brain.[253] The ophthalmic manifestations of myopathies have recognised importance as potential biomarkers,[254] and in DMD the retina characteristics may offer a portal to the CNS.

Two boys with severe neurocognitive profile could only manage the GOSH skin protocol. This technique offers a tolerable, child-friendly assessment of retinal physiology if the neurobehavioural profile is severely affected, which is often the case in DMD, especially in boys with mutations at the 3'end of the gene.

In conclusion, these data further refine the characteristics of the retinal function of DMD. The frequently reported electro-negative scotopic ERG associates with mutations downstream of exon 30 and is profound when Dp71 is additionally

affected. Selective stimulation of slow and fast rod pathways shows a dependence of slow rod pathways upon Dp260. Also we have highlighted anomalies of the amacrine oscillatory potential circuits, and cone pathways associated with Dp427 and Dp71. The most severe b-wave changes reflect the most severe neurocognitive profile and likely reflect abnormal dystrophin isoform CNS expression.

Our study has several limitations: the sample size was small, therefore these results need to be explored in larger cohort of patients especially with mutations in the 3'-end of the gene; we have speculated that our electrophysiological findings are secondary to dystrophin disruption in the retina, however further studies are required to understand the underlying signalling mechanisms in humans, which determine these electrophysiological abnormalities, and to what extent they can be reversed; finally, any relationship between neurodevelopmental problems and retinal electrophysiological abnormalities requires further systematic investigation in larger cohort of patients.

In conclusion, while further investigating the intricate and yet poorly understood role of dystrophin and its isoforms, retinal electrophysiology could offer a non-invasive portal into understanding protein disruption and potential restoration in the CNS.

CHAPTER 6: NUCLEAR MAGNETIC RESONANCE INVESTIGATIONS IN DMD

The work contained in this chapter has given rise to the following publication:
Ricotti V, Evans MRB, Sinclair CDJ, Butler JW, Ridout DA, Hogrel JY, Emira A, Morrow JM, Reilly MM, Hanna MG, Janiczek RL, Matthews PM, Yousry TA, Muntoni F and Thornton JS. *Evaluation of upper limb in Duchenne muscular dystrophy: fat-water quantification by MRI, muscle force and function define endpoints for clinical trials.* (Under review)

My contribution to this work consisted of the following:
In collaboration with the Institute of Myology, UCL and GSK I oversaw the design and analysis of the study. I contributed in the development of the upper limb modules used to asses patients in this study. I recruited patients. I contributed to data collection. In collaboration with the Statistical Department I analysed the data. I wrote the first draft of the manuscript, and contributed to the revision of the manuscript.

Imaging and the muscle

The use of imaging for the assessment of muscle disease raised interest in the neuromuscular community when in 1980 Heckmatt, Dubowitz and Leeman carried out a pilot study on use of muscle ultrasound, which was published on the Lancet.[255] Subsequently the same group reported the appearance of the quadriceps muscle of the thigh in 60 children affected by different neuromuscular conditions and in 60 control children. The authors could show a very good visualization of bone echo, which stood out clearly against the

background of echo-free muscle tissue, whilst muscle of boys with muscular dystrophies was associated with an increase in the intensity of echo reflected from the muscle tissues, with corresponding loss of bone echo. Changes in muscle echo were also reported in children with congenital myopathies, whilst children with non-neuromuscular conditions, such as hypotonia, had normal findings.

Ultrasound imaging has proved a very useful, non-invasive screening tool in the investigation of children with neuromuscular disease, and it is currently widely used in clinics to identify muscle involvement, and also as an aid in the selection of muscle biopsy sites. In the recent years magnetic resonance imaging (MRI) and spectroscopy (MRS) have rapidly increased in importance in the neuromuscular field both in assisting the diagnostic pathway but also as very promising outcome measures in experimental studies.

The advantage conferred by MR technology is that it is a non-invasive method without ionizing radiation, with high resolution of muscle fat and connective tissue, and capable of measuring tissue characteristics (MRI) and muscle metabolites (MRS) in a quantitative way.

MRI works by creating around the area to be imaged, a strong magnetic field, which can range from 1 to 7 tesla. Hydrogen atoms in tissues containing water molecules are used to create a signal that is processed to form an image of the target tissue or organ. First, energy from an oscillating magnetic field is temporarily applied to the patient at the appropriate resonance frequency. The excited hydrogen atoms emit a radio frequency signal, which is measured by a receiving coil. The radio signal can be made to encode position information by varying the main magnetic field using gradient coils. The contrast between

different tissues is determined by the rate at which excited atoms return to the equilibrium state.

MRS is also a non-invasive biochemical sampling technique, which can be used to infer further information about cellular activity to quantify certain metabolites in the target tissues such as lipids or ATP in skeletal muscle.

MRI in neuromuscular diseases

The use of MRI largely contributed to shift from a diagnostic approach based on muscle biopsy to clinical and MRI pattern recognition in support of a targeted genetic investigation.[256] Imaging has shown how different muscle diseases affect muscles selectively, and consistently, therefore creating a disease distribution pattern, which facilitates diagnosis. A TREAT-NMD workshop held in Rome in 2011 summarised the characteristic findings for the different muscle diseases.[257] The MRI muscle study group in addition extensively reported pattern recognition in early onset neuromuscular disorders with full body MRI protocols.[258]

MRI studies in Duchenne muscular dystrophy

For Duchenne muscular dystrophy, numerous studies have shown the ability of MRI to detect alterations in skeletal muscles and its composition.[256, 259-274] Matsuma *et al.* in 1988 reported T1 values in DMD to rapidly decrease with time as disease progresses, secondary to replacement of muscle by fat.[260] The group also showed that the pattern of T1 reduction was not the same for all muscles, as it was most prominent in the gluteus maximus and least prominent in the sartorius and gracilis muscles. The authors reported that both regenerating and

degenerating muscle fibres showed an increased muscle water content, resulting in high T1 value in the early stages of DMD, whereas in the advanced stages of the condition replacement of muscle by fat caused decrease in T1 values.[259] Another pioneering study reported increased T2 values of anterior tibial muscle in DMD patients when compared to healthy controls, and this was explained by the increased fatty infiltration in DMD patients.[261] Furthermore, in weaker DMD patients, the width of the muscle T2 peak increased and the peak shifted toward the fat peak. In addition, the T1 values decreased with increasing fatty infiltration. The authors concluded that quantitative T1 and T2 maps could be used to assess muscle status and monitor DMD progression.[261]

Mercuri *et al*.[256] subsequently developed a six step staging system to qualitatively characterise disease severity based on visual inspection of the skeletal muscle, the so called "*Mercuri score*":

- **Stage 0:** normal appearance
- **Stage 1:** Scattered small areas of increased density
- **Stage 2a:** numerous discrete areas of increased density ≤ 30% of the volume of the muscle
- **Stage 2b:** Numerous discrete areas of increased density with early confluence, 30-60% of the volume of the muscle
- **Stage 3:** Numerous perimysial and endomysial adipocytes with loss/partial loss of fascicular structure
- **Stage 4:** Pronounced fatty replacement throughout with fascicle structure loss.

Although this score was recently used to screen DMD subjects recruited into a clinical trial for local administration of antisense oligomer nucleotides,[77] and was

also used in a comparative study of muscle histology with MRI, its limitation is that it relies on quality inspection rather than quantification of muscle pathology.[262]

A number of quantitative methodologies have been applied to DMD including: T1-weighted imaging,[259] T1-weighted imaging and gadolinium enhancement,[263] muscle cross sectional area;[264] T2-weighted imaging;[256, 265, 268, 273, 275] Fat suppression sequences;[266] 3-Point Dixon technique;[267, 269, 270] and finally MR Spetroscopy.[268, 271, 272]

As an example, T1-weighted (T1W) with gadolinium contrast-enhanced images have been used to quantify the differences between normal controls and corticosteroid-treated DMD boys in the lower limb muscles and to explore the effect of exercise.[263] Eight of nine muscles studied showed a significant increase in T1-W signal intensity in DMD as compared to normal muscle, suggestive of increased fat infiltration in DMD muscle. The DMD boys were also imaged before and after stepping exercise: and as exercise effect an increased gadolinium uptake was observed only in the tibialis anterior muscle.[263]

T1-weighted images at 3 time points were used to assess fat replacement in the thighs of 8 DMD ambulant boys on corticosteroids.[267] This study showed that as early as 9 months and over 18 months the fat replacements in the thigh muscle increases significantly, especially in the biceps femoris, rectus femoris and vastus lateralis. This study proved MRI to be able to demonstrate the specific fat changes over time and also the variability between different muscle groups.[267]

Another study focused on T2-weighted imaging to establish quantifiable measures, which would allow correlation with clinical evaluations and with the "*Mercuri score*" assessment.[265] The study, which was carried out on the upper leg of 34 DMD boys (mean age of 8.5 years) quantified the degree of muscle

involvement by T2 mapping methodologies. The study showed that the highest T2 values, reflecting fat degeneration, were observed in the gluteus maximus, and these values correlated with the Mercuri scores, as well as with clinical assessment (i.e. time rising from the floor and the 30 feet run).[265]

A large multi-centre cross-sectional study has been recently published evaluating MRI and MRS in the lower extremities of 123 DMD boys and 31 healthy controls.[268] This study supported the validity and reproducibility of an imaging protocol across centres;[276] furthermore it showed how MR-T_2 measures of the lower limb muscles are sensitive at differentiating DMD patients who are still ambulant from healthy controls, and these values deteriorate with age and pathology progression.

Dixon technique

The Dixon technique has been originally been described by Thomas Dixon, who in 1984 proposed a chemical shift imaging capable of separating MRI signal intensity for the distinct contributions of water and fat.[277] In his original technique two sets of spin echo images were acquired with slightly different echo times: the first with fat and water signals in phase and the second with the echo times adjusted by a few milliseconds so that the fat and water signals were out-of-phase. This technique results in high-resolution water and fat maps. During the 1990s numerous modifications of the Dixon technique were proposed, including 3-point methods:[278, 279] this requires three gradient echo images at three different echo times to be acquired with the echo times determined by the field strength of the magnet.[258] Usually two of the acquisitions are done with water and fat in phase whilst a third acquisition is out of phase. This technique has

been recently used by Wren and colleagues[269] in nine boys with DMD, MRI measures of fat infiltrations in the thigh were compared with functional grades according to the Brooke Scale[280] showing a strong correlation between the 2 parameters. More recently the lower limbs of 16 DMD patients and 11 controls were imaged with the 3-point Dixon technique.[270] This study described fat fraction percentage values in all the muscle groups of the upper and lower leg, with values significantly higher than in controls. The mean age of the DMD patients was 11.4 years (± 2.2 y.), 7 of them fully ambulant and the others wheelchair users/dependant. The most affected muscles included abductor magnus, biceps femoris (long head and rectus femoris, with f.f.% in the range of 40-60. Furthermore this study demonstrated that although the total cross sectional area of the muscles did not differ significantly between controls and DMD, on the other hand, the contractile muscle cross sectional area was significantly smaller in DMD, particularly the quadriceps femoris and hamstrings. Maximal voluntary isometric contraction was obtained using a fixed myometry. All muscle groups showed decreased strength when compared to controls.[270]

MR Spetroscopy

In recent years various approaches of MR spectroscopy (MRS) have been explored to study muscle pathology in DMD. Although MRS focuses on a limited area of the muscle, it can provide accurate information on the muscle metabolites. ^{31}Phosphorus spectroscopy (^{31}P MRI) has been reported in patients with DMD[281, 282] and has been evaluated and described in in the soleus and tibialis anterior muscles of healthy subjects by Kan *et al*.[271] The idea behind ^{31}P

MRI is that it can be used to measure cellular energy in organs; to assess mitochondrial content and function in skeletal muscles; and to evaluate muscle perfusion and acidification.[283]

^{31}P MRI, and most specifically phosphodiesters to phosphocreatine (PDE/PCr), a single sensitive index for membrane metabolism analysis, have been recently used to describe improvement in muscles of Golden Retriever Muscular Dystrophy (GRMD) dogs treated with AVV-delivered exon skipping, alongside histopathology and muscle strength.[154]

Furthermore, proton spectroscopy (^{1}H-MRS) has been evaluated in muscles. Torriani *et al*.[272] measured ^{1}H-MRS lipid fraction in the soleus and tibialis anterior of 9 DMD subjects and 8 matched healthy controls. In this study a relationship between muscle lipid composition and the 6-minute walk test (6MWT) was observed. The same study found a positive correlation between ^{1}H-MRS measures which were part of the imaging protocol and the 10 metre run. [272] However, these correlations need to be interpreted with caution given the cross-sectional nature of the study and the small sample size.

In their large cross-sectional multicentre study involving 123 ambulant DMD and 31 healthy controls, alongside MRI-T_2, Forbes *et al.* described also ^{1}H-MRS measures acquired for the assessment of lipid fraction in the lower extremities. The fat fraction, which is given by the ratio lipid/(lipid + water) was significantly higher in DMD subjects and as for the MRI-T_2 measures, these values increased with age,[268] therefore providing a potential biomarker for monitoring disease progression and therapeutic intervention.

MRI as an endpoint for clinical trials

MRI continues to be largely investigated both in animal models,[154, 284] in natural history studies [275, 285] and in interventional studies.[286, 287]

International collaborations supported by TREAT-NMD have facilitated the standardization of operating procedures and the harmonization of protocols for clinical trials in DMD.[258] Furthermore, a multicentre study carried out in the United States has validated MRI and MRS protocols for DMD ambulant subjects.[276]

As discussed above, MRS has been explored as a biomarker sensitive to response to pharmaco-genetic therapy in GRMD dogs.[154] A recent study evaluated both MRI and MRS of the legs in response to steroid therapy.[286] MRI and MRS measures of fat infiltration favoured DMD subjects on steroids when examined cross-sectionally; in addition imaging detected therapeutic effects of steroid treatment in reducing inflammation over the course of one year. With a number of potential therapeutic interventions under development for DMD, MRI/MRS seem to offer potential non-invasive and non-motivational-dependent biomarkers for monitoring disease progression and response to treatment.

Muscle biopsy is currently used to demonstrate dystrophin expression in clinical trials that aim at restoring truncated yet functional protein products. [78, 79, 82] However, this is an invasive procedure, which carries the risks of exposing the subjects to general anaesthetics and can only be carried out a limited number of times in the same subjects. A study by Kinali *et al.* showed a correlation between MRI changes based on the *Mercuri* scoring system evaluated by two observers, and histopathologic changes on muscle biopsy of the extensor digitorum brevis.[262]

MRI not only allows to evaluate muscle pathology non-invasively but also to evaluate an overall progression of the pathology. It is well recognised that DMD pathology varies between muscles and within the same muscle, dystrophin expression may not be uniform.[288] Hence, MRI may offer a more global view of the pathology.

As discussed in chapters 3, a number of promising novel experimental therapies are at different stages of clinical trials, e.g. antisense oligomer (AO) mediated exon skipping, which aims to restore semi-functional protein products; and drugs to induce read-through of nonsense mutations.[78, 79, 82] The 6-minute-walk test (6-MWT) has been utilized as a primary outcome end-point in phase II-III clinical trials;[80-82] however limitations of this functional test include that it is motivation dependent[289] and can only be used for ambulant boys. As discussed above, recent studies showed that MRI and MRS provide sensitive markers of muscle pathology and disease progression in the lower limbs of DMD subjects,[267, 268, 273] however to date very limited data exists about upper limb imaging in non-ambulant subjects.

A study on evaluation of upper limb in Duchenne muscular dystrophy: fat-water quantification by MRI, muscle force and function define endpoints for clinical trials.

The aim of my study was to examine the ability of MRI, specifically MRI fat quantification, to detect disease progression in the upper limb of non-ambulant DMD boys and to compare the responsiveness of quantitative MRI measures to muscle strength and validated functional assessments.

Methods

Participants

I recruited 15 non-ambulant DMD boys with a mean age of 13.3 y, (range: 10.8-17.3 years) mean duration of non-ambulation 20.2 months (range: 4.7- 41.6) and mean BMI 26.5, range: 20.8-41.7). Assessments were performed at baseline, 3, 6 and 12 months; 6 subjects withdrew from the study due to the frequent hospital visits, which were challenging to sustain in absence of therapeutic intervention. At the time of the study, all but one subject were receiving glucocorticoids at the time of the study. Ten age and gender-matched healthy control subjects were scanned once (mean age: 14.6y, range: 13-17; mean BMI 21.5, range: 16.5-25.4). To assess measurement reproducibility 4 of these individuals were re-tested at 6 weeks.

Approval from the local research ethics committee was obtained for this study, which was performed in compliance with the Declaration of Helsinki.

MRI acquisition and data analysis

Unilateral upper-limb MRI was performed at 3T (Siemens, Erlangen, Germany) using a flexible surface matrix coil (4-Channel Flex Coil) wrapped around the dominant forearm. Subjects lay in the scanner in the head-first supine position, with the dominant arm to be imaged lying in a comfortable position on the scanner bed alongside the torso. Three-point-Dixon[278] images were acquired. Images were post-processed offline with a Python programming language pipeline according to Glover and Schneider's algorithm[279] and separated fat (f) and water (w) images were used to calculate pixel-wise fat fraction (f.f.) maps according to f.f.(%)= 100*f/(w+f).

Observers blinded to diagnosis manually defined individual muscle-group regions of interest (ROI) on the TE=3.45ms Dixon image to encompass each muscle-group boundary to the fascia using the ITK Snap software[290]. For consistency all ROI definitions were subsequently reviewed by a single blinded observer (based at the Institute of Neurology). For each subject 3 of the 9 available baseline image slices were chosen for analysis: a central slice defined as the first axial slice distal to the supinator muscle, and proximal and distant slices centred ± 74mm relative to the central slice. For consecutive longitudinal data from the same subject, the slices selected for analysis were carefully matched to the baseline slices by visual inspection and reference to a coronal scout image. On each slice ten forearm muscle-group ROIs were defined: the (extensor carpi ulnaris (ECU), extensor digiti minimi (EDM), extensor digitorum (ED), extensor pollicis longus (EPL), abductor pollicis longus (APL), extensor carpi radialis longus/brevis and the brachioradialis (ECRLB Br), flexor digitorum profundus and flexor pollicis longus (FDP), flexor digitorum superficialis and palmaris longus (FDS), flexor carpi ulnaris (FCU), flexor carpi radialis (FCR) muscle/muscle groups (figure 29).

Figure 29. Muscle segmentation and raw 3-point Dixon of the central slice in the dominant forearm of a healthy control

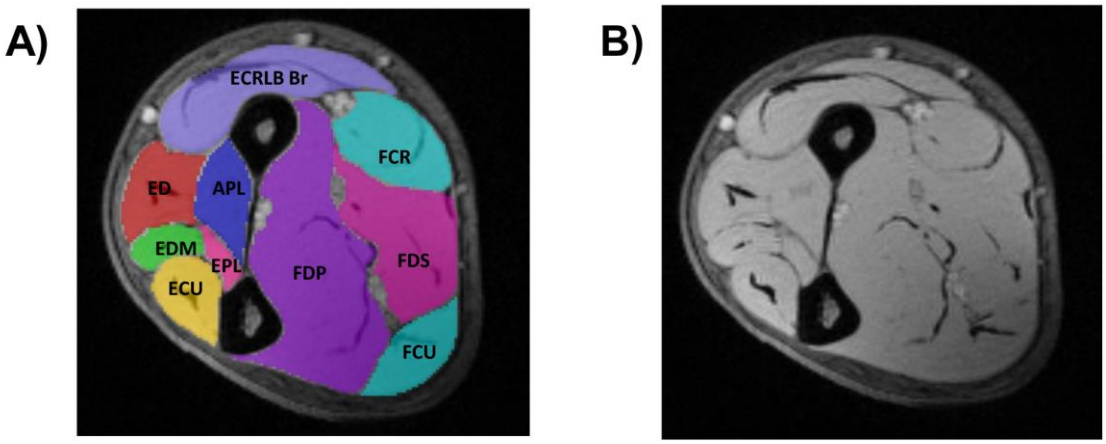


Figure 29. Muscle segmentation (A) and raw 3-point Dixon (B) of the central slice in the dominant forearm of a healthy control **.**

Extensor carpi ulnaris (ECU), extensor digiti minimi (EDM), extensor digitorum (ED), extensor pollicis longus (EPL), abductor pollicis longus (APL), extensor carpi radialis longus/brevis and brachioradialis (ECRLB Br), flexor digitorum profundus and flexor pollicis longus (FDP), flexor digitorum superficialis and palmaris longus (FDS), flexor carpi ulnaris (FCU), flexor carpi radialis (FCR).

For each consecutive image at the same level in the same subject, ROI were drawn with reference to the baseline ROI, to maintain equivalent anatomical coverage. The segmented ROI were transferred to the co-registered f.f. maps, and visually inspected for placement errors or the presence of gross artefact and adjusted if necessary. For all individual ROIs, custom-written software extracted individual muscle-group mean f.f. and muscle cross-sectional area (CSA) in mm^2, and also calculated for each subject at each of the 3 slice levels the summary measures *overall muscle f.f.* calculated as the mean of the individual muscle-group mean f.f.s weighted by their respective CSAs, *total cross-sectional muscle area* calculated as the sum of the individual muscle CSAs, and *total remaining muscle area* calculated as *total cross-sectional muscle area* (100 – *overall muscle %f.f.*)/100.

Functional assessment

Each subject was assessed as follows: 1) performance of upper limb (PUL) module, a validated 74 point functional scale for motor performance relating to everyday life activity;[291, 292] 2) muscle strength for shoulder flexion, elbow extension and flexion, and wrist extension was measured in lbs using a hand-held myometer (Microfet, Hoggan, UT) 3) MyoSet (Myopinch, Myogrip and Moviplate), a suite of recently validated novel tools to assess strength and fatigability of the upper limb;[293, 294] 4) Egen Klassification (EK2) interview version 2, which is used to determine performance of tasks in daily life (total score=51)[295] 5) spirometry and peak cough flow by using Vitalograph Pneumotrac 6800; and 6) the time to loss of ambulation (LOA) in months was also recorded.

Statistical methods

Baseline MRI measures were compared between the DMD and control groups using unpaired t-tests. In the DMD group, changes in clinical measurements and MRI scores were compared within boys over the 3 follow-up time points, using mixed model regression analysis. A separate model was fitted for each outcome measurement and an adjustment was made for length of time non-ambulant; results are presented as adjusted mean changes for the 3 month, 6 month and 12 month periods with 95% confidence intervals (CI). The Normality assumption of the models was checked using the Shapiro-Francia W dash test.[128] The Shapiro-Francia normality test performs a formal test of Normality for a single variable. The null hypothesis of the test assumes the population is normally distributed and a resulting significant p value would suggest there is evidence of non-Normality. [128] For measurements where there was some evidence of non-normality we investigated the use of appropriate transformations. [128] Findings were found to be very similar therefore we have presented results for untransformed data. To account for multiplicity a P-value < 0.01 was considered significant. The statistics package *Stata*[132] was used for the analysis.

Results

Out of the 15 subjects initially recruited, combined MRI and clinical assessment data were obtained for 9/15 subjects at 3 month (mean age 13.2y; range: 11.1-17.6) follow-up and 7/15 at both 6 the month (mean age 13.9y; range: 11.6-17.9) and 12-month (mean age 14.2y; range: 11.8-18.2) time points. Although MRI was overall well tolerated, the remaining 6 subjects found the commitment to the study too demanding to sustain.

Muscle MRI- fat infiltration, cross-sectional area, and remaining non-fat area

The overall mean central slice muscle f.f. (95% CI) in the DMD subjects was significantly higher than for healthy controls (14.1%, 95% CI 9.1, 19.1 in DMD; 0.9% 95% CI 0.8, 1.0 in healthy controls; $p<0.001$), as were individual muscle-group mean f.f. (table 18). The central slice mean total cross-sectional muscle area was reduced in DMD (1473 mm^2, 95% CI 1289.3, 1654.7 mm^2) relatively to the healthy controls (2389 mm^2, 95% CI 1878.9, 2899.0 mm^2; $p=0.006$). The extensor carpi radialis longus/brevis and the brachioradialis (ECRLB Br) group showed the greatest differences in f.f. between DMD and the healthy controls (table 18), with an overall mean f.f. 31.1 (95% CI 22.5; 39.7%) in the DMD group.

Table 18 (A&B). Individual muscle-group mean fat fraction (%) for the central slice in each DMD and healthy control (HC) subject at baseline (A) and mean (95%CI) changes from baseline (B).

A

Subjects	ECU f.f.	EDM f.f.	ED f.f.	APL f.f.	EPL f.f.	ECRLB Br f.f.	FCU f.f.	FDP f.f.	FCR f.f.	FDS & PL f.f.
1 DMD	3.41	1.14	3.50	4.58	3.40	26.41	5.88	4.99	4.90	7.09
2 DMD	5.44	2.95	5.27	10.98	9.49	55.10	E	12.94	26.15	46.72
3 DMD	8.56	12.69	9.86	19.26	7.56	13.36	49.10	16.69	24.19	34.17
4 DMD	2.25	3.45	4.03	4.48	5.81	43.67	11.64	2.65	5.16	6.18
5 DMD	1.73	2.44	4.47	3.21	4.10	9.76	22.81	7.74	34.73	13.39
6 DMD	2.87	1.23	1.87	1.93	2.43	14.58	3.82	1.32	4.19	5.81
7 DMD	2.21	7.72	40.36	25.28	22.94	54.52	6.97	35.21	24.01	47.72
8 DMD	91.92	88.59	68.19	84.76	75.76	46.23	75.94	91.95	87.61	94.56
9 DMD	0.77	0.00	0.03	0.29	0.66	7.84	3.97	1.58	0.48	1.52

10 DMD	7.94	14.24	17.23	16.11	13.13	52.27	31.68	11.07	30.58	12.68
11 DMD	12.69	11.53	25.37	23.47	30.19	30.69	32.30	22.78	38.58	47.92
12 DMD	2.57	2.24	3.68	2.01	2.25	22.12	6.03	1.93	21.74	15.85
13 DMD	2.15	3.18	4.93	4.96	5.93	22.34	6.40	3.66	6.92	1.99
14 DMD	5.39	11.54	12.56	25.14	12.51	32.43	12.08	3.61	27.84	11.97
15 DMD	12.96	13.33	24.29	35.66	19.79	37.66	36.40	27.24	56.73	41.59
Subjects	**ECU f.f.**	**EDM f.f.**	**ED f.f.**	**APL f.f.**	**EPL f.f.**	**ECRLB Br f.f.**	**FCU f.f.**	**FDP f.f.**	**FCR f.f.**	**FDS & PL f.f.**
1 HC	0.09	0.47	1.50	0.72	0.00	1.67	2.42	0.50	1.34	0.93
2 HC	1.48	0.63	0.95	0.28	1.40	1.03	1.58	0.59	0.07	0.24
3 HC	0.13	0.52	1.01	0.53	1.19	1.20	0.20	0.11	0.46	0.58
4 HC	1.14	0.68	2.07	0.64	0.68	0.78	2.41	0.58	1.84	0.88
5 HC	4.37	1.39	4.39	0.06	-1.55	4.70	0.40	0.16	0.01	0.01
6 HC	2.35	1.18	2.47	0.19	0.99	1.31	2.25	0.73	2.04	1.82
7 HC	0.66	0.80	0.98	0.48	0.38	0.25	3.31	0.44	0.91	0.93
8 HC	1.66	0.60	1.65	0.14	3.58	4.30	0.85	0.86	0.65	0.61
9 HC	1.69	1.31	1.23	0.98	2.09	1.50	1.15	0.37	2.03	0.68
10 HC	1.22	1.04	1.01	0.70	0.34	1.34	-0.07	0.57	0.57	0.38

B

		3 months	6 months	12 months
ECU FAT FRACTION (%)				
	Mean change from baseline (95% CI)	0.7 (-0.3, 1.8)	0.7 (-0.4, 1.8)	1.6 (0.6, 2.7)
	No. of subjects	9	7	8
	P value	0.17	0.19	<0.01
EDM FAT FRACTION (%)				
	Mean change from baseline (95% CI)	0.1 (-2.2, 2.5)	2.2 (-0.4, 4.6)	3.8 (1.4, 6.3)
	No. of subjects	9	7	8
	P value	0.92	0.10	<0.01
ED FAT FRACTION (%)				
	Mean change from baseline (95% CI)	1.2 (-0.7, 3.2)	2.3 (0.2, 4.4)	2.9 (0.9, 4.9)
	No. of subjects	9	7	8
	P value	0.21	0.03	<0.01
APL FAT FRACTION (%)				
	Mean change from baseline (95% CI)	1.2 (-2.1, 4.5)	4.3 (0.8, 7.9)	6.6 (3.2, 10.0)
	No. of subjects	9	7	8
	P value	0.48	0.02	<0.001
EPL FAT FRACTION (%)				
	Mean change from baseline (95% CI)	1.2 (-0.8, 3.2)	1.0 (-1.1, 3.1)	1.3 (-0.8, 3.3)
	No. of subjects	9	7	8
	P value	0.23	0.35	0.22
ECRLB Br FAT FRACTION (%)				
	Mean change from baseline (95% CI)	1.7 (-1.1, 4.5)	6.1 (3.1, 9.2)	7.0 (4.1, 10.0)
	No. of subjects	9	7	8
	P value	0.24	<0.001	<0.001
FCU FAT FRACTION (%)				
	Mean change from baseline (95% CI)	3.6 (0.6, 6.6)	3.9 (0.6, 7.1)	6.0 (2.9, 9.1)
	No. of subjects	9	7	8
	P value	0.02	0.02	<0.001

FDP FAT FRACTION (%)				
	Mean change from baseline (95% CI)	0.05 (-2.4, 2.5)	1.3 (-1.3, 3.9)	4.8 (2.3, 7.3)
	No. of subjects	9	7	8
	P value	0.97	0.32	<0.001
FCR FAT FRACTION (%)				
	Mean change from baseline (95% CI)	2.0 (-1.9, 5.9)	2.6 (-1.6, 6.8)	7.1 (3.1, 11.1)
	No. of subjects	9	7	8
	P value	0.31	0.22	<0.01
FDS & PL FAT FRACTION (%)				
	Mean change from baseline (±SD)	2.7 (0.3, 5.0)	6.0 (3.5, 8.6)	7.1 (4.7, 9.6)
	No. of subjects	9	7	8
	P value	0.03	<0.001	<0.001

Table 18: Extensor carpi ulnaris (ECU), extensor digiti minimi (EDM), extensor digitorum (ED), abductor pollicis longus (APL), extensor pollicis longus (EPL), extensor carpi radialis longus/brevis and brachioradialis (ECRLB Br), flexor carpi ulnaris (FCU), flexor digitorum profundus and flexor pollicis longus (FDP), flexor digitorum; flexor carpi radialis (FCR); superficialis and palmaris longus (FDS & PL). P value < 0.01 was considered significant.

Longitudinally we observed a significant increase in central slice overall muscle f.f. at 6 months, with a mean increase of 3.8% (95% CI 1.9, 5.7%) above baseline in 7 DMD ($p<0.001$). At 12 months the mean increase for central slice overall muscle f.f. was 5.0 (95% CI 3.2, 6.9) above baseline (n=7). Examples of 3-point Dixon images are shown on figure 30. Group mean changes from baseline are reported on table 19. Individual trajectories of DMD subjects are shown in figure 30a.

Proximal and distal slice mean increases also were significant at 12 months: 6.5% (95%CI 3.9, 8.9%; $p<0.001$) and 4.3% (95%CI 2.0, 6.6%; $p<0.001$) respectively (table 19, figure 31 b&c).

At 12 months, the dorsal compartment was more affected than the volar compartment, with, at the central-slice level, a mean increase of 6.3 % (95%CI 3.6, 9.1; $p<0.001$) compared to the volar compartment (3.4 %, 95% CI 0.9, 5.7; $p<0.01$) (table18). All individual muscle groups showed a significant increase in f.f. over a year (all $p<0.001$, e-table 1b) with the ECRLB Br remaining the most affected muscle group, with a mean increase of f.f. equal to 7.0% (95% CI 4.1, 10.0; $p<0.001$) (table18 B)

Mean central slice total cross-sectional muscle area in the DMD subjects increased above baseline by 140 mm^2 (95% CI 50.9, 229.1 mm^2) at 12 months ($p<0.01$); however the total remaining (i.e. non-fat) muscle area, which at baseline was 1472 mm^2 (95% CI 1289.3, 1654.7 mm^2) remained overall unchanged at 12 months (mean change in 7 subjects was = 22.0 mm2, 95% CI -48.2, 92.3 mm^2, $p=0.5$).

The mean f.f. (95% CI) central slice overall mean muscle f.f. change between baseline and 6 weeks in healthy controls was 0.8% (95% CI 0.6, 0.9%; $p=0.67$).

Figure 30. Three-point Dixon fat-fraction maps of dominant forearm central slice at baseline and 12 months

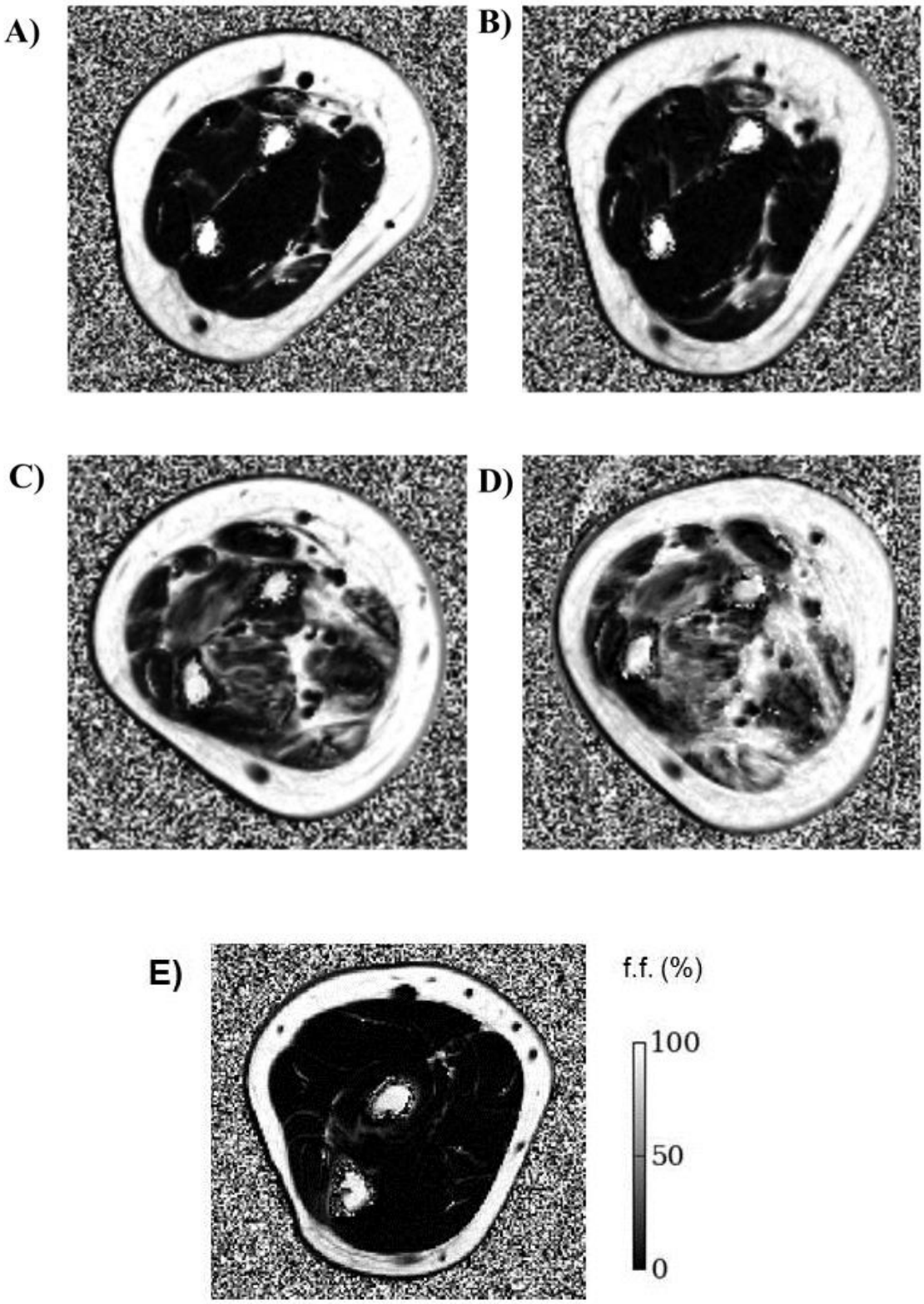


Figure 30. 3-point Dixon fat-fraction (f.f.) maps of dominant forearm central slice at baseline (left images) and 12 months (right images) of DMD subjects (A-D) and healthy control (E). Normal muscle appears black, in contrast with fat which is white, for example the sub-cutaneous fat and bone marrow. In DMD patients, fat infiltrated muscles are also white. Fat-fraction maps allow fat quantification (expressed in %, weighted for the area) in the regions of interest of the muscles which are evaluated.
Top (A&B): 13 y.o. DMD, non-ambulant for 40 months, and on daily steroids. Overall mean f.f. at baseline= 7.6% (A) and 12 months = 9.7% (B). **Middle (C&D):** 11 y.o. DMD, non-ambulant for 14 months, who was discontinued from steroid therapy. Mean f.f. at baseline= 30.7%(C) and 12 months =43.3%(D). (Grey-level bars represent f.f. from 0 to 100%). **Bottom (E):** gender-matched healthy control (13 years), mean f.f.= 0.7%

Table 19. MRI and clinical indices mean changes from baseline from analysis of variance.

	3 months	6 months	12 months
CENTRAL SLICE TOTAL MUSCLE COMPARTMENT FAT FRACTION (%)			
Mean change from baseline (95% CI)	1.4 (-0.3, 3.2)	3.9 (1.9, 5.7)	5.0 (3.2, 6.9)
No. of subjects	9	7	7
P value	0.11	< 0.001	< 0.001
PROXIMAL SLICE TOTAL MUSCLE COMPARTMENT FAT FRACTION (%)			
Mean change from baseline (95% CI)	2.1 (0.5, 3.7)	4.5 (2.7, 6.3)	6.9 (5.2, 8.7)
No. of subjects	9	6	7
P value	0.01	< 0.001	< 0.001
DISTAL SLICE TOTAL MUSCLE COMPARTMENT FAT FRACTION (%)			
Mean change from baseline (95% CI)	2.18 (0.06, 4.29)	2.23 (-0.05, 4.52)	4.29 (2.01, 6.58)
No. of subjects	9	7	7
P value	0.04	0.06	< 0.001
CENTRAL SLICE DORSAL COMPARTMENT FAT FRACTION (%)			
Mean change from baseline (95% CI)	1.7 (-0.9, 4.4)	5.5 (2.7, 8.3)	6.3 (3.6, 9.1)
No. of subjects	9	7	8
P value	0.20	<0.001	<0.001
CENTRAL SLICE VOLAR COMPARTMENT FAT FRACTION (%)			
Mean change from baseline (95% CI)	1.3 (-1.0, 3.7)	0.7 (-1.8, 3.3)	3.4 (0.9, 5.7)
No. of subjects	9	7	8
P value	0.27	0.58	<0.01
ECRLB Br FAT FRACTION (%)			
Mean change from baseline (95% CI)	1.7 (-1.1, 4.5)	6.1 (3.1, 9.2)	7.0 (4.1, 10.0)
No. of subjects	9	7	8
P value	0.24	<0.001	<0.001

CENTRAL SLICE TOTAL MUSCLE COMPARTMENT CROSS-SECTIONAL MUSCLE AREA (mm^2)				
	Mean change from baseline (95% CI)	-5.4 (-88.1, 77.4)	42.1 (-47.0, 131.2)	140 (50.9, 229.1)
	No. of subjects	9	7	7
	P value	0.90	0.36	< 0.01
CENTRAL SLICE TOTAL REMAINING (NON-FAT) MUSCLE AREA (mm^2)				
	Mean change from baseline (95% CI)	-28.4 (-93.6, 36.83)	-32.1 (-102.6, 38.1)	22.0 (-48.2, 92.28)
	No. of subjects	9	7	7
	P value	0.39	0.37	0.54
MYOPINCH (Kg)				
	Mean change from baseline (95% CI)	-0.1 (-0.27, 0.03)	-0.38 (-0.53, -0.22)	-0.50 (-0.66, -0.34)
	No. of subjects	10	9	8
	P value	0.12	< 0.001	< 0.001
MYOGRIP (Kg)				
	Mean change from baseline (95% CI)	-0.22 (-0.70, 0.27)	-0.50 (-1.01, 0.002)	-1.04 (-1.56, -0.51)
	No. of subjects	10	9	8
	P value	0.38	0.05	< 0.001
PERFORMANCE OF UPPER LIMB (Total score=74)				
	Mean change from baseline (95% CI)	-0.5 (-2.8, 1.9)	-2.6 (-5.2, -0.03)	-9.2 (-11.9, -6.4)
	No. of subjects	14	11	9
	P value	0.69	0.05	< 0.001
PERFORMANCE OF UPPER LIMB (Shoulder domain score =16)				
	Mean change from baseline (95% CI)	-0.7 (-2.6, 1.1)	-1.0 (-2.9, 0.9)	-6.9 (-9.0, -4.9)
	No. of subjects	14	11	10
	P value	0.42	0.32	< 0.001
MOVIPLATE (taps in 30 seconds)				
	Mean change from baseline (95% CI)	2.3 (-0.02, 4.6)	4.7 (2.01, 7.4)	1.7 (-0.8, 4.3)
	No. of subjects	11	7	8
	P value	0.05	0.001	0.19

Table 19. MRI and clinical indices mean changes (95%CI) from baseline from analysis of variance. P value <0.01 was considered significant

Figure 31. Plots of individual trajectories

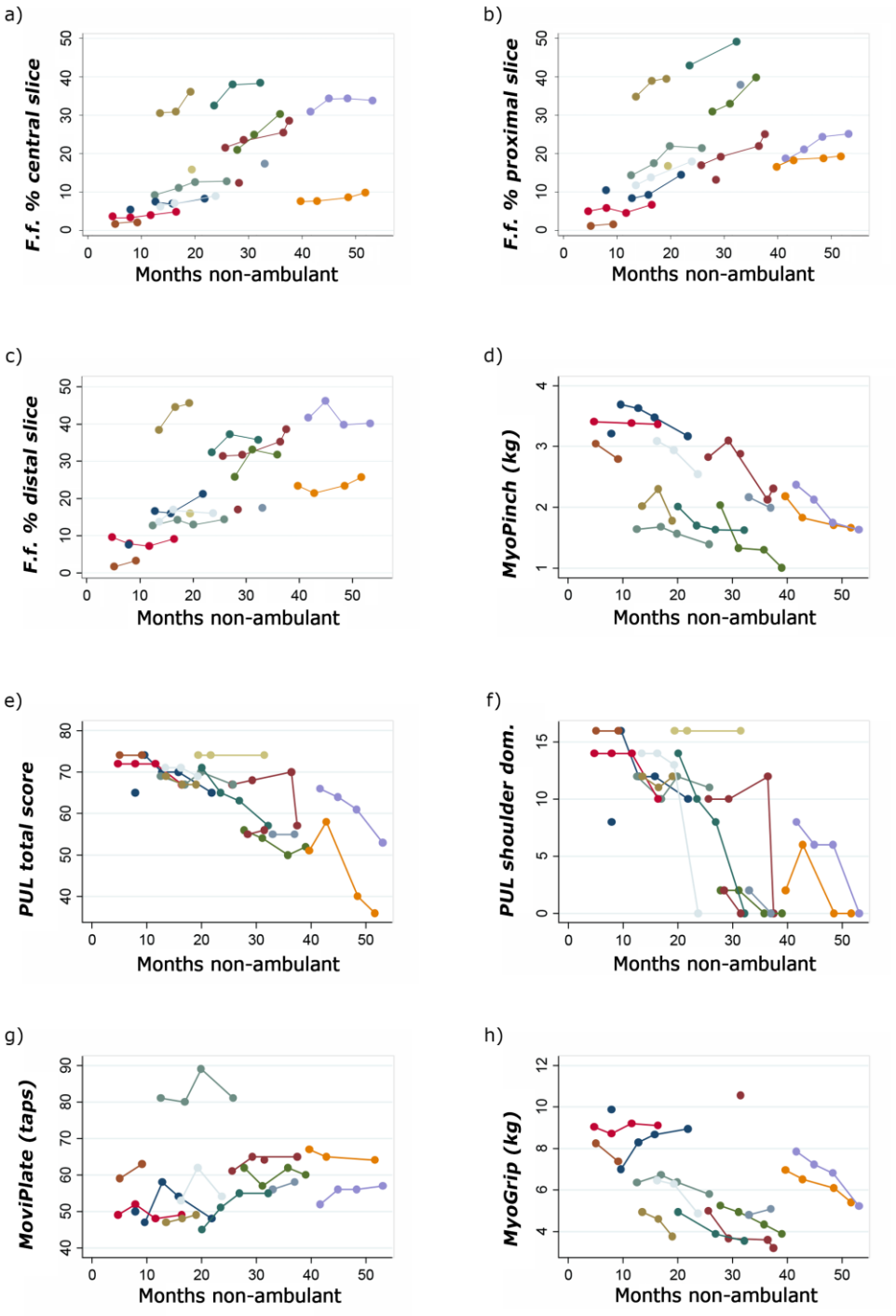


Figure 31. Plots of individual trajectories for (A) central slice overall muscle %fat fraction, (B) proximal slice overall muscle %fat fraction, (C) distal slice overall muscle %fat fraction, (D) MyoPinch, (E) total score for Performance of Upper

limb, (F) PUL Shoulder domain score, (G) MoviPlate and(H) MyoGrip. F.f.= fat fraction; PUL= Performance of upper limb

Excluding the DMD boy who was not receiving steroids from the analysis did not change the statistical conclusions (table 20).

Table 20. **MRI and clinical indices: mean changes from baseline; analysis of variance excluding the DMD subject not taking steroid therapy.**

		3 months	6 months	12 months
CENTRAL SLICE TOTAL MUSCLE COMPARTMENT FAT FRACTION (%)				
	Mean change from baseline (95% CI)	1.40 (0.06, 2.73)	3.53 (2.07, 4.98)	3.90 (2.45, 5.35)
	No. of subjects	8	6	6
	P value	0.04	< 0.001	< 0.001
PROXIMAL SLICE TOTAL MUSCLE COMPARTMENT FAT FRACTION (%)				
	Mean change from baseline (95% CI)	1.79 (0.30, 3.26)	4.46 (2.81, 6.10)	6.03 (4.42, 7.64)
	No. of subjects	8	5	6
	P value	0.02	< 0.001	< 0.001
DISTAL SLICE TOTAL MUSCLE COMPARTMENT FAT FRACTION (%)				
	Mean change from baseline (95% CI)	1.71 (-0.30, 3.71)	1.63 (-0.55, 3.81)	3.20 (1.02, 5.38)
	No. of subjects	8	6	6
	P value	0.10	0.14	< 0.01
CENTRAL SLICE DORSAL COMPARTMENT FAT FRACTION (%)				
	Mean change from baseline (95% CI)	1.5 (-1.4, 4.3)	5.0 (1.9, 8.1)	5.4 (2.5, 8.4)
	No. of subjects	8	6	7
	P value	0.32	<0.01	<0.001
CENTRAL SLICE VOLAR COMPARTMENT FAT FRACTION (%)				
	Mean change from baseline (95% CI)	1.3 (-0.6, 3.3)	0.06 (-2.0, 2.2)	2.1 (0.1, 4.1)
	No. of subjects	8	6	7
	P value	0.18	0.95	0.04
ECRLB Br FAT FRACTION (%)				
	Mean change from baseline (95% CI)	1.3 (-1.6, 4.1)	4.8 (1.8, 7.9)	5.9 (3.0, 8.8)
	No. of subjects	8	6	7
	P value	0.38	<0.01	<0.001

CENTRAL SLICE TOTAL MUSCLE COMPARTMENT CROSS-SECTIONAL MUSCLE AREA (mm2)				
	Mean change from baseline (95% CI)	-17.93 (-99.68, 63.83)	18.15 (-70.70, 106.97)	101.47 (12.65, 190.29)
	No. of subjects	8	6	6
	P value	0.67	0.69	0.03
CENTRAL SLICE TOTAL REMAINING (NON-FAT) MUSCLE AREA (mm2)				
	Mean change from baseline (95% CI)	-38.65 (-110.08, 32.79)	-46.26 (-123.86, 31.34)	15.19 (-62.41, 92.79)
	No. of subjects	8	6	6
	P value	0.29	0.24	0.70
MYOPINCH (Kg)				
	Mean change from baseline (95% CI)	-0.16 (-0.31, -0.004)	-0.39 (-0.55, -0.23)	-0.52 (-0.68, -0.36)
	No. of subjects	9	8	8
	P value	0.04	< 0.001	< 0.001
MYOGRIP (Kg)				
	Mean change from baseline (95% CI)	-0.20 (-0.72, 0.32)	-0.43 (-0.97, 0.12)	-1.01 (-1.56, -0.47)
	No. of subjects	9	8	8
	P value	0.45	0.13	< 0.001
PERFORMANCE OF UPPER LIMB (Total score=74)				
	Mean change from baseline (95% CI)	-0.48 (-2.84, 1.88)	-2.60 (-5.18, -0.03)	-9.19 (-11.96, -6.42)
	No. of subjects	13	10	8
	P value	0.69	0.05	< 0.001
PERFORMANCE OF UPPER LIMB (Shoulder domain score =16)				
	Mean change from baseline (95% CI)	-0.37 (-2.79, 2.05)	-2.70 (-5.37, -0.03)	-8.37 (-11.26, -5.48)
	No. of subjects	13	10	8
	P value	0.77	0.05	< 0.001

MOVIPLATE (taps in 30 seconds)				
	Mean change from baseline (95% CI)	2.42 (-0.05, 4.90)	5.08 (2.14, 8.02)	1.86 (-0.82, 4.55)
	No. of subjects	10	6	8
	P value	0.06	0.001	0.17

Table 20. MRI and clinical indices: mean (95%CI) changes from baseline; analysis of variance excluding the DMD subject not taking steroid therapy. P value < 0.01 was considered significant.

Clinical assessment

At 6 months mean MyoPinch-measured pinch force decreased significantly from baseline by 0.38 kg (95% CI -0.22, -0.53; p<0.01) (table 19, figure 31e) in the DMD group (n=8). Grip force measured by MyoGrip in 8 DMD subjects, significantly decreased at 12 months from baseline by 1.0 kg (95% CI -1.56, -0.51) over the duration of the study (p<0.001). The group average PUL total score and shoulder domain index deteriorated significantly at 12 months, with a mean drop of 9.2 (95% CI -11.9; -6.4; p<0.001) and –6.9 (95% CI -9.0; -4.9; p<0.001) respectively. Lower arm functional assessment using the MoviPlate detected a significant improvement of function at 6 months (4.69, 95% CI 2.0, 7.4; p=0.001) with subsequent decline (table19, figure 31 g). These conclusions remain unchanged by excluding the subject not treated with steroids (table 20). All other evaluations, including spirometry and muscle strength measurement with myometry, failed to reach statistical significance. Individual trajectories of some of these measures are shown in figure 32.

Figure 32. Plots of individual trajectories

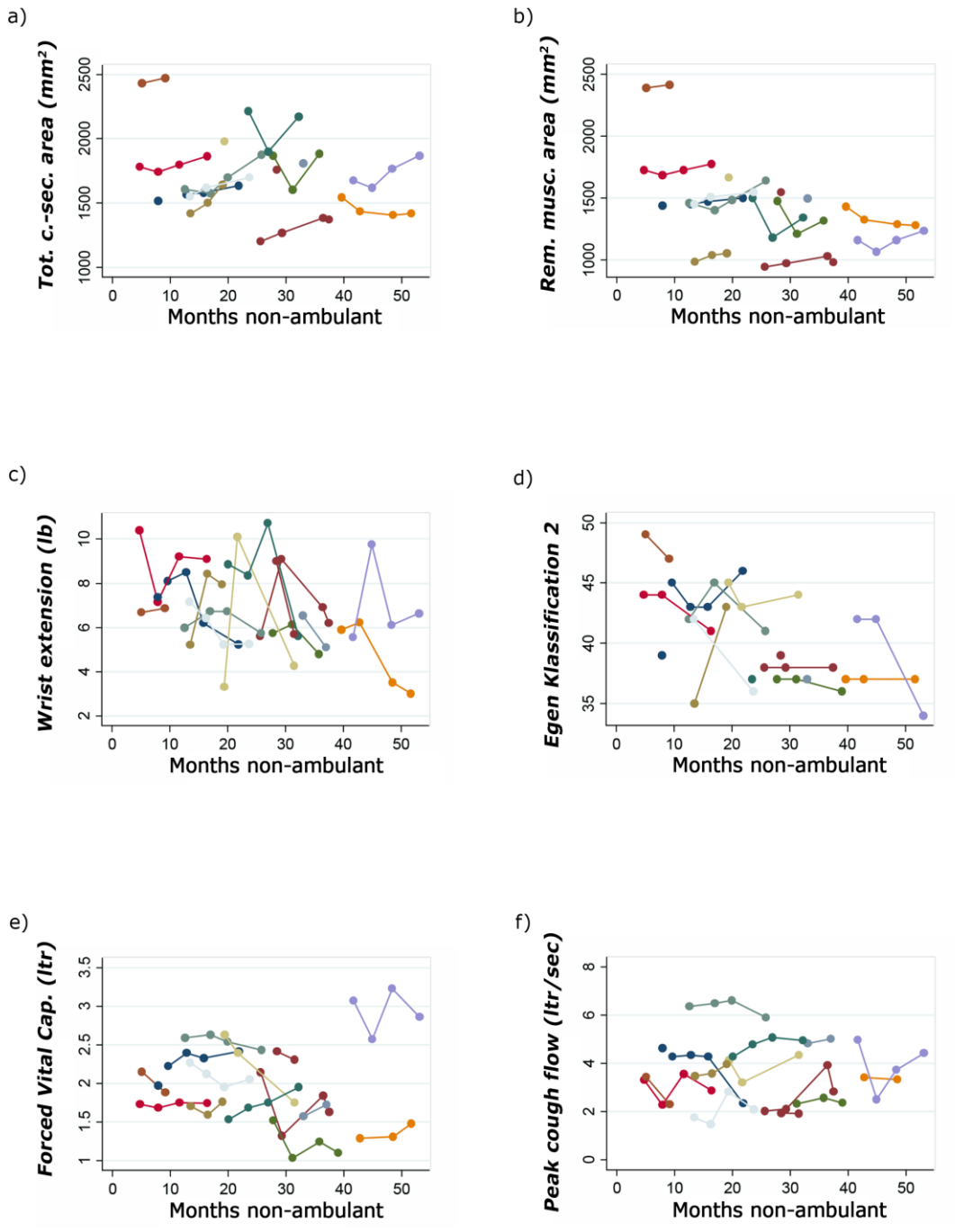


Figure 32: (A) Individual trajectories for central slice total cross sectional muscle area in mm2 and (B) total remaining muscle area in mm2 , (C) wrist extension with microfet myometer, (D) Egen Klassification (EK2), (E) Forced vital capacity (ltr) (F), Peak cough flow(ltr/sec)

Discussion

This study suggests that MRI fat quantification in the upper limb forearm is a sensitive biomarker for disease progression in non-ambulant DMD boys. Not only was MRI capable of discriminating DMD from healthy controls (p=0.002), but it also allowed detection of increased forearm muscle-fat infiltration in the dominant forearm of DMD subjects as early as 6 months from baseline (p<0.001). At one year, the DMD group mean central slice muscle f.f. increase was 5.0% (95% CI 3.2, 6.9%) above a baseline value of 14.1% (95% CI 9.1, 19.1%; p<0.001). In addition, we demonstrated that whilst in the patient group mean total muscle compartment cross-sectional area increased over 12 months, the remaining total non-fatty muscle area within the muscle compartments did not change. We speculate that this reflects the process of fat infiltration or transformation in DMD, which, in the lower limb has been associated with hypertrophic changes. All muscle groups showed a significant increase in f.f. over one year (all p<0.001), with the dorsal compartment more affected than the volar. The ECRLB Br was the most severely affected muscle group at baseline (mean f.f. 31.1, 95% CI 22.5; 39.7%) and progressed more rapidly over the course of the study with a mean f.f. increase of 7.0% (95% CI 4.1, 10.0; p<0.001) at 12 months. The fat transformation of muscle extended over the whole length of the lower arm: proximal and distal slices showed a similar relative progression of fat transformation, although the proximal slice was more severely affected. Even within individual muscles, fat transformation showed more rapid progression in the more proximal segments.

In relation to the clinical evaluation, at 6 months we observed significant decrease from baseline in pinch strength of 0.38 kg (95% CI -0.22, -0.53; p<0.001) as measured by the MyoPinch. This was sustained at one year (-0.50 kg 95% CI -

0.22, -0.53 kg from baseline, p<0.001). Over 12 months handgrip strength was also significantly reduced by 1.0 kg (95% CI -1.56, -0.51 p<0.001). In line with previous publications,[293, 294] the MoviPlate, a tapping device designed to measure endurance of upper limb, showed a modest improvement over 6 months followed by a decline. This phenomenon has been observed in the early non-ambulant phases (i.e. boys who are wheelchair-bound < 3years)[294] and can possibly be explained by a functional training effect of the distal upper limb following loss of ambulation and increase demands on arm function. The functional scale PUL showed highly significant disease progression and loss of function at 12 months (p<0.001), with a mean drop of 9.2 scores (95% CI -11.9; -6.4) out of 74; this effect was predominantly driven by the loss of function in the shoulder domain. Interestingly, the loss of shoulder-girdle muscle function is associated to a progressive fatty transformation of lower arm muscles, suggesting that the changes we observed are not limited to the forearm muscles. Muscle strength of specific muscle groups was measured including shoulder extension, elbow flexion and extension, and wrist extension; however these assessments did not yield any significant results within the time frame studied and in this relatively small cohort of patients. None of the other assessments, including spirometry and a patients' reported questionnaire showed significant changes over 12 months.

When designing clinical trials for rare disorders, critical issues need to be taken into account and these include: selecting an endpoint which is as observer and subject-motivation independent as possible; reducing the burden of participation in clinical trials favouring non-invasive endpoints; and selecting an endpoint capable of identifying clinically meaningful changes in a small cohort of patients. MRI technologies are increasingly applied to neuromuscular disorders, not only

in defining phenotype[257], but also in providing biomarkers responsive to therapies.[286, 287] Studies have already demonstrated the suitability of MRI to characterise pathology in DMD, [268, 296] monitor disease progression in the lower limbs [267, 273, 297] and in the upper limb of non-ambulant subjects.[285] Further studies have explored imaging in combination with strength and/or functional assessment of the lower extremities.[270, 274, 298-301]A recent study has shown that MRI and MRS can also effectively detect the beneficial effect of glucocorticoid therapy both in cross-sectional comparison and at 3 months.[286] Furthermore, MRI combines the advantages of being overall well tolerated: unlike muscle biopsy, it is not an invasive procedure, and also allows the simultaneous quantitative assessment of multiple muscles.

Workshops were held by international experts under the auspices of the TREAT-NMD with the objective to harmonize the protocols for MR-based outcome measures in skeletal muscle studies.[258] Moreover, a multicentre study evaluated the reproducibility among centres of MRI and MRS in lower limbs of DMD boys,[276] facilitating recruitment in multicentre studies. In recent studies the 3-point-dixon technique[278, 302] has been evaluated as possibly the best methodology to be able to globally measure fat transformation in DMD,[258, 269, 270] since fat transformation has been associated with disease progression in DMD, also supported by muscle pathology. Indeed, MRI and MRS have been included as exploratory endpoints in clinical trials for ambulant boys in patients treated with Drisapersen[287] and in the on-going EU FP7 funded SKIP-NMD programme, which aims to restore dystrophin with morpholino antisense oligomer exon skipping for patients with deletions skippable by exon 53, and which will yield results by 2016.[287, 303]

To my knowledge, our pilot study provides longitudinal data on fat transformation in the forearm of non-ambulant DMD with 3-point Dixon imaging in the context of detailed clinical evaluation measurements for the first time. MRI and Myopinch sensitively detected changes at 6 months and in that case they both qualify as endpoints; however, these are highly complementary endpoints. MRI allows assessing individual muscles, and allows a comprehensive view across different muscle groups at different locations, whereas Myopinch specifically measures a function of strength, which reflects a joint effort of one distinct group of muscle. Furthermore, our results support the notion that f.f. determined by MRI could provide a reliable objective and clinically-meaningful marker for disease monitoring in DMD. The progression in fat transformation as measured by MRI in our cohort resulted not only in a decline in strength (for pinching at 6 months, and gripping at 12 months), but also in a functional impairment as measured by the PUL at 1 year; we therefore conclude that the increase in f.f. is clinically meaningful.

The high sensitivity for change in DMD, which can detect significant disease progression as early as 6 months in a small cohort of patients, would allow design of clinical trials with shorter duration and limit the need for the larger sample sizes associated with conventional clinical end-points. For example, in our study we observed a mean change in total cross-sectional muscle f.f. at 6 months from baseline of approximately 4% (± 3%) in 7 DMD subjects. In clinical trials, if this change was compared with an expected mean change of approximately 0.5% in a treated group,[286] assuming a similar SD, a power of 90% and significance level of 5%, a minimum of 16 patients per group would be required. This would considerably reduce the sample size and duration of the study compared to studies designed with, for example, the 6-MWT as a clinical

end-point. Furthermore, these data also supports the feasibility of recruiting non-ambulant DMD subjects into clinical trials under the assumption that a response to treatment should also be observed in the upper limb.

This study protocol was well tolerated overall, allowing adequate rest between the image acquisition and physiotherapy assessment. The 6 subjects who withdrew from the study reported that the frequent hospital visits (i.e. 4 in 12 months) were difficult to sustain in absence of therapeutic intervention. Although cooperation in lying still in the scanner is indispensable for image acquisition, on the whole this is not regarded as motivational-dependent as for conventional endpoints such the 6-MWT, for which performance may be affected by the level of tiredness or motivation on the day.

Limitations of this study include the sample size being small, with a selected population (i.e. non-ambulant and below 18 years of age). It is likely that the measures explored in our study may vary according to age at baseline and length of time non ambulant, and it would be of interest to adjust for these factors in a future larger study. Furthermore, although it was not our objective here to explore the effect of treatment including steroids on MRI response, this may be important. However, excluding from the analysis the single patient not on steroid therapy did not alter our broad findings. Finally, our MRI coverage was limited to the dominant forearm, and the fat transformation analysis to three slices; further studies should address pathology and disease progression in limb regions and muscle groups not included here.

In conclusion, the results of our study provide novel data to inform rational imaging endpoint incorporation and trial design for studies in non-ambulant Duchenne cohorts. I documented objective changes in MRI measures of muscle fatty transformation and suggested their clinical meaningfulness by

demonstrating a strong correlation with measures of muscle force, function and disease progression. The time course of response to deterioration observed suggests detection of disease stabilization or improvements over periods as short as 6-12 months is readily achievable. The complementarity of the methods suggests a mechanistic relationship between fat transformation of muscle tissues, muscle force production and overall function.

CHAPTER 7: FINAL DISCUSSION AND CONCLUSIONS

Challenges of clinical trial design for DMD

The changing natural history of DMD

The natural history of Duchenne Muscular Dystrophy (DMD) is undoubtedly a shifting target, as shown by the work of my thesis and reported by numerous recent studies.[29, 35, 304] And by "*natural history*" it is intended DMD-with-all-the-intervention-available-to-date, which are summarised and elucidated in the standard of care documents published by Bushby and colleagues.[32, 33] The natural history of this disorder goes hand in hand with the on-going evolution of therapies; hence this phenomenon needs to be respected and factored into the design of clinical trials. Applying metrics based on old knowledge of the condition without taking into account new interventions risks making clinical trial design poorly adapted or obsolete and recruitment into studies very challenging. For example, most ambulant patients are now on glucocorticoid therapy, and the age of starting steroids is indeed shifting towards a younger age as shown in the UK clinical practice;[305] hence, designing studies with steroid therapy as an exclusion criterion in the young ambulant population risks making recruitment virtually impossible in most specialised centres. This could in turn skew recruitment and introduce recruitment biases. Furthermore, cardiac medications used prophylactically (i.e. beta blockers and ACE-inhibitors) are increasingly supported by evidence that their use is beneficial for DMD patients,[52, 53, 306] although a consensus on what is the optimal age for commencing therapy is lacking. An on-going 5-year long clinical trial funded by the British Heart Foundation testing ACE-inhibitor (perindopril) combined with beta-blocker therapy (bisoprolol) is targeting DMD boys 7-12 years of age who have

not developed signs of cardiomyopathy (EudraCT number: 2007-005932-10). This study will likely strengthen the body of literature in support of early intervention[51] and indeed prevention; hence clinical trial design will have to become permissive towards heart medication given prophylactically. However, at least in animal models medications targeting angiotensin not only impact on cardiac load, but appear also beneficial for skeletal muscle[307] making it difficult to assess if such medications introduce a bias into skeletal muscle-based trial outcome. A similar scenario will apply to anti-oxidative treatment such as ibedenone; with the recently published study[146] supporting the beneficial effect on respiratory function it is expected that soon enough many patients will be treated on ibedenone alongside steroids although the study results were obtained on steroid-naïve patients and it is unknown to what degree the two drugs are complementary.

Targeting the multisystem nature of DMD

While current efforts are mainly focused on improving the skeletal muscle function, and some also cardiac function, it is becoming increasingly clear that , the evolving therapeutic interventions for DMD should ideally also reflect the multisystem nature of this disorder. This requires a shift in thinking: the target of experimental therapies should therefore not be solely the muscle but should ideally include other tissues and organs, such as smooth muscle, and the brain, also affected in this disorder. Such interventions are indeed under development, for example with novel chemistries such as the modified antisense oligonucleotides peptide conjugated morpholinos (PPMO) and the tricyclo-DNA, not only target efficiently skeletal and cardiac muscle but also the brain in animal

models.[308, 309] If proven to be beneficial in patients, these therapies have the potential of prolonging life span even further, as currently the most important cause of premature mortality in DMD is cardiomyopathy. Furthermore it is conceivable that therapies could also ameliorate the emotional/behavioural problems associated with disrupted dystrophin protein products in the brain. Indeed systemic administration of the tricyclo-DNA chemistry showed complete correction of behavioural features in the treated *mdx* mouse, yielding promising perspectives for future intervention also in humans; some of the most recently developed PPMOs also appear to be effective in crossing the blood brain barrier (M. Wood, F. Muntoni, M. Gait, personal observation). If proven to be beneficial in patients, these novel class therapies have the potential of not only prolonging life span even further, as currently a very important cause of premature mortality in DMD is cardiomyopathy, but could potentially also address some of the issues related to deficiency of dystrophin in the CNS.

The spectrum of phenotype within DMD

The current clinical trials and natural history studies have also brought to surface further heterogeneity in the DMD population. For example, when plotting a functional scale score such as the North Star Ambulatory Assessment versus age it becomes evident that the motor function varies even in a cohort of patients assessed and treated with uniform protocols.[159, 168, 305] A wide phenotypical spectrum is also a feature in other multisystem manifestations of DMD, such as the neuro-cognitive profile and is strongly influenced by the genotype [71, 72, 310, 311] Similarly, response to treatment may vary from one subject to the other, including the response to corticosteroid therapy.[35] With this in mind, in-depth

zoom on the inter-individual variability also begged the question: what are the determinants of variable disease course and manifestation? It was described by the work of my thesis [305] and recent publications [168, 169] that different *DMD* mutations can follow a variable course, showing that genotype bears some role in phenotype expression. Even within cohorts amenable to exon skipping those deletions skippable by exon 44 clearly follow a milder course than those skippable by exons 51 or 53. Although these differences may only become evident after 2 years of observation, clinical trial design needs to account for such variability. Novel techniques of dystrophin quantification [288, 312-314] have recently allowed to accurately quantifying the resulting protein product also across laboratories.[315] This has facilitated the use of dystrophin quantification as a treatment-response-related biomarker in the setting of clinical trials; [76-81] furthermore it allowed also to understand the role that even low residual protein expression may have in contributing to a less severe phenotype.[162, 170, 316] Recently described gene disease modifiers also play a role in prolonging ambulation[174, 175] and influence treatment response to steroids.[172] However, these results may need to be reproduced and further explored in larger scale populations, as their specific role especially in different ethnicities still needs to be fully evaluated. Finally, in spite of the efforts made to standardize treatment approaches such as steroid therapy, different regimens are currently being used and therapy is introduced at different ages, impacting on the effect that steroids can have on the disease course during the ambulant phase[35] but possibly also after the age of loss of ambulation.[44, 317]

Impact of clinical trial design and outcome measures.

With all these considerations in mind the design of clinical trials for DMD needs to take the dynamic variables of this condition into account. In such a panorama, biomarkers play a very important role. Biomarkers can be used to demonstrate pharmacodynamics proof of concept, as for example dystrophin quantification in dystrophin replacement therapies. Biomarkers can also be used to stratify patients, for example genetic disease modifiers (e.g. *LTBP4, SPP1*)[172-175, 318] could guide stratification of less/more severely affected subjects if cohorts are sufficiently large. In addition, imaging biomarkers, such as the assessment of fatty transformation of skeletal muscle by MRI and MRS can be implemented to explore the variability of the phenotype and in future to create more homogenous cohorts for clinical trials. Finally, these novel biomarkers can potentially be used to monitor treatment response. For-example, fragments of myomesin-3, a myofibrillar structural protein, which are measured at abnormal levels in the sera of DMD subjects, have been found to be sensitive to monitor response to therapy in in *mdx* mice treated with morpholino AON.[319] Similarly, longitudinal data on serum matrix metalloproteinase-9 showed increased levels with age and disease progression in DMD patients.[320] In relation to the brain, monitoring of the bioelectrical response of the retina by ERG may be a convenient and non-invasive biomarker for monitoring the effects of CNS dystrophin restoration in retinal neurons.[321] This is also pertinent for AAV-based gene correction therapy because AAV9 has previously been shown to effectively transduce the retina after systemic injection.[322] A recent publication [154] reported improvement in skeletal muscle MR indices in Golden Retriever Muscular Dystrophy dogs injected with AVV- mediated U7snRNA-coupled antisense sequences promoting exon skipping. The injected limb after three months

showed improvement in T2w intensity; ^{31}P NMS demonstrated a decrease in phosphodiester (PDE) signal and decrease in phosphocreatine (PCr) signal. The PDE/PCr ratio has been described to characterise muscle membrane metabolism and could potentially represent an effective biomarker of sarcolemma integrity.[283] Moreover, it was recently reported that the determination of the skeletal muscle fat fraction by MRI/MRS was able to detect the beneficial effects of steroid therapy in DMD subjects within 3 months of treatment.[286]

MRI and MRS are currently included as an exploratory endpoints in clinical trial protocols for Duchenne Muscular dystrophy (http://www.skip-nmd.eu) and are able to monitor the relentlessly progressive fatty transformation, now documented for lower [267, 268] and upper extremities [285] as also described in the work of my thesis. These can be used as a baseline ruler of the disease progression. It is foreseeable that in future MRI/MRS may become a primary endpoint for clinical trials in DMD and other myopathies.

The use of poly-therapies and a place for registries

In neuromuscular disorders, specifically DMD, we are now most certainly entering an era when speeding-up of therapies is required. Clinical trial design needs to be adapted to the new disease course, and novel outcome measures need to be developed and implemented to reflect such an evolution. It is predictable that within the next decade the treatment approaches for DMD will substantially alter the disease course; at the same time none of the approaches under development will cure the disease and likely this will be the scenario 10 years from now. It is also predictable that medications now developed as single therapy need to be combined for a successful therapeutic approach in DMD (e.g.

gene therapy, AON, steroids, anti-oxidative medication). However concepts for developing combined therapeutic protocols such as in cancer treatment are still largely lacking for DMD and time-scales of decades are simply not acceptable. Of course the time pressure of a rapid malignancy versus that of a chronic progressive handicapping disease is different, hence fuelling different strings of research. This is why regularly and systematically updated registries and national databases may serve as useful platforms to capture the effect of combined treatment approaches and thereby modernise medicine even more so for rare and chronic disorders. National registries/database will be able to fulfil demands that are unlikely to be fulfilled by industry-sponsored studies. A successful example is the NorthStar clinical network and database in the UK, which allowed addressing questions such as long-term effects of different steroid regimens before any clinical trials could do;[35] furthermore it advised the UK neuromuscular community on bone protection guidelines and on the disease course of different mutations.[305] Likewise, the US-based clinical network CINRG and the Italian clinical network provided valuable and informative long-term natural history data on large cohorts of ambulant and non-ambulant subjects [43, 116, 117, 159, 160] and also allowed to define disease-influencing genetic modifiers polymorphisms.[318] I envisage that in the future similar and larger platforms could offer the opportunity to address other questions, for example in relation to poly-therapy (e.g. combined idebenone and steroids in the ambulant population) or the optimal age of starting cardiac medication prophylactically, or long term side effects of dystrophin replacement therapy (e.g. PTC). The use of national databases to define impact of therapeutic strategies today remains not readily accepted for regulatory purposes, with the exception of post-marketing follow up. However, industry is frequently not willing to finance trials for preventive

treatment where follow-up longer than 5 years may be necessary to document a treatment effect, notably for medications already in use for common disorders. Furthermore, industry is even less willing to document cumulative positive or negative poly-treatment effects where part of the cocktail is conflicting with their interest. In this emerging panorama national and international databases/registries can play a critical role for the advancement of therapies and their development in DMD.

Final conclusion

In conclusion, with DMD as an example, I illustrated that multiple and combined strategies are required to accelerate therapeutic developments for neuromuscular disorders. This should include disease-specific and -sensitive outcome measures, which advance hand in hand with the evolving natural history of the condition; clinical trial design, which takes into account the variables and dynamics of the disorder; and finally integrate through intelligent use of registries/databases the collection of broad-based evidence to strengthen knowledge building and modernise clinical care for DMD.

REFERENCES

1. Mah JK, Korngut L, Dykeman J, Day L, Pringsheim T, Jette N. A systematic review and meta-analysis on the epidemiology of Duchenne and Becker muscular dystrophy. Neuromuscul Disord 2014;24:482-491.
2. Theadom A, Rodrigues M, Roxburgh R, et al. Prevalence of muscular dystrophies: a systematic literature review. Neuroepidemiology 2014;43:259-268.
3. Emery AE. Population frequencies of inherited neuromuscular diseases--a world survey. Neuromuscul Disord 1991;1:19-29.
4. Mendell JR, Shilling C, Leslie ND, et al. Evidence-based path to newborn screening for Duchenne muscular dystrophy. Ann Neurol 2012;71:304-313.
5. Ellis JA, Vroom E, Muntoni F. 195th ENMC International Workshop: Newborn screening for Duchenne muscular dystrophy 14-16th December, 2012, Naarden, The Netherlands. Neuromuscul Disord 2013;23:682-689.
6. Moat SJ, Bradley DM, Salmon R, Clarke A, Hartley L. Newborn bloodspot screening for Duchenne muscular dystrophy: 21 years experience in Wales (UK). Eur J Hum Genet 2013.
7. Mendell JR, Lloyd-Puryear M. Report of MDA muscle disease symposium on newborn screening for Duchenne muscular dystrophy. Muscle Nerve 2013;48:21-26.
8. Bushby KM, Hill A, Steele JG. Failure of early diagnosis in symptomatic Duchenne muscular dystrophy. Lancet 1999;353:557-558.
9. Mohamed K, Appleton R, Nicolaides P. Delayed diagnosis of Duchenne muscular dystrophy. Eur J Paediatr Neurol 2000;4:219-223.
10. Marshall PD, Galasko CS. No improvement in delay in diagnosis of Duchenne muscular dystrophy. Lancet 1995;345:590-591.
11. Connolly AM, Florence JM, Cradock MM, et al. Motor and cognitive assessment of infants and young boys with Duchenne Muscular Dystrophy: results from the Muscular Dystrophy Association DMD Clinical Research Network. Neuromuscul Disord 2013;23:529-539.

12. Pane M, Scalise R, Berardinelli A, et al. Early neurodevelopmental assessment in Duchenne muscular dystrophy. Neuromuscul Disord 2013;23:451-455.
13. De Sanctis R, Pane M, Sivo S, et al. Suitability of North Star Ambulatory Assessment in young boys with Duchenne muscular dystrophy. Neuromuscul Disord 2015;25:14-18.
14. Melis MA, Cau M, Congiu R, Puddu R, Muntoni F, Cao A. Germinal mosaicism in a Duchenne muscular dystrophy family: implications for genetic counselling. Clin Genet 1993;43:247-249.
15. Prior TW, Bridgeman SJ. Experience and strategy for the molecular testing of Duchenne muscular dystrophy. J Mol Diagn 2005;7:317-326.
16. Dent KM, Dunn DM, von Niederhausern AC, et al. Improved molecular diagnosis of dystrophinopathies in an unselected clinical cohort. Am J Med Genet A 2005;134:295-298.
17. Flanigan KM, von Niederhausern A, Dunn DM, Alder J, Mendell JR, Weiss RB. Rapid direct sequence analysis of the dystrophin gene. Am J Hum Genet 2003;72:931-939.
18. Muntoni F, Torelli S, Ferlini A. Dystrophin and mutations: one gene, several proteins, multiple phenotypes. Lancet Neurol 2003;2:731-740.
19. Tuffery-Giraud S, Beroud C, Leturcq F, et al. Genotype-phenotype analysis in 2,405 patients with a dystrophinopathy using the UMD-DMD database: a model of nationwide knowledgebase. Hum Mutat 2009;30:934-945.
20. Bladen CL, Salgado D, Monges S, et al. The TREAT-NMD DMD Global Database: analysis of more than 7,000 Duchenne muscular dystrophy mutations. Hum Mutat 2015;36:395-402.
21. Muntoni F. Is a muscle biopsy in Duchenne dystrophy really necessary? Neurology 2001;57:574-575.
22. Flanigan KM, Dunn DM, von Niederhausern A, et al. Mutational spectrum of DMD mutations in dystrophinopathy patients: application of modern diagnostic techniques to a large cohort. Hum Mutat 2009;30:1657-1666.

23. Aartsma-Rus A, Van Deutekom JC, Fokkema IF, Van Ommen GJ, Den Dunnen JT. Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. Muscle Nerve 2006;34:135-144.
24. Blake DJ, Weir A, Newey SE, Davies KE. Function and genetics of dystrophin and dystrophin-related proteins in muscle. Physiol Rev 2002;82:291-329.
25. Pietri-Rouxel F, Gentil C, Vassilopoulos S, et al. DHPR alpha1S subunit controls skeletal muscle mass and morphogenesis. Embo J 2010;29:643-654.
26. Brenman JE, Chao DS, Xia H, Aldape K, Bredt DS. Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. Cell 1995;82:743-752.
27. Muntoni AEaF. Duchenne Muscular Dystrophy, III ed: Oxford University Press, 2003.
28. Eagle M, Bourke J, Bullock R, et al. Managing Duchenne muscular dystrophy--the additive effect of spinal surgery and home nocturnal ventilation in improving survival. Neuromuscul Disord 2007;17:470-475.
29. Ishikawa Y, Miura T, Aoyagi T, Ogata H, Hamada S, Minami R. Duchenne muscular dystrophy: survival by cardio-respiratory interventions. Neuromuscul Disord 2011;21:47-51.
30. Moxley RT, 3rd, Pandya S, Ciafaloni E, Fox DJ, Campbell K. Change in natural history of Duchenne muscular dystrophy with long-term corticosteroid treatment: implications for management. J Child Neurol 2010;25:1116-1129.
31. Eagle M, Baudouin SV, Chandler C, Giddings DR, Bullock R, Bushby K. Survival in Duchenne muscular dystrophy: improvements in life expectancy since 1967 and the impact of home nocturnal ventilation. Neuromuscul Disord 2002;12:926-929.
32. Bushby K, Finkel R, Birnkrant DJ, et al. Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. Lancet Neurol 2010;9:77-93.

33. Bushby K, Finkel R, Birnkrant DJ, et al. Diagnosis and management of Duchenne muscular dystrophy, part 2: implementation of multidisciplinary care. Lancet Neurol 2010;9:177-189.
34. Manzur AY, Kinali M, Muntoni F. Update on the management of Duchenne muscular dystrophy. Arch Dis Child 2008;93:986-990.
35. Ricotti V, Ridout DA, Scott E, et al. Long-term benefits and adverse effects of intermittent versus daily glucocorticoids in boys with Duchenne muscular dystrophy. J Neurol Neurosurg Psychiatry 2013;84:698-705.
36. Scott OM, Hyde SA, Goddard C, Dubowitz V. Prevention of deformity in Duchenne muscular dystrophy. A prospective study of passive stretching and splintage. Physiotherapy 1981;67:177-180.
37. McDonald CM. Limb contractures in progressive neuromuscular disease and the role of stretching, orthotics, and surgery. Phys Med Rehabil Clin N Am 1998;9:187-211.
38. Siegel IM. Plastic-molded knee-ankle-foot orthoses in the treatment of Duchenne muscular dystrophy. Arch Phys Med Rehabil 1975;56:322.
39. Smith AD, Koreska J, Moseley CF. Progression of scoliosis in Duchenne muscular dystrophy. J Bone Joint Surg Am 1989;71:1066-1074.
40. Alman BA, Raza SN, Biggar WD. Steroid treatment and the development of scoliosis in males with duchenne muscular dystrophy. J Bone Joint Surg Am 2004;86-A:519-524.
41. Lebel DE, Corston JA, McAdam LC, Biggar WD, Alman BA. Glucocorticoid treatment for the prevention of scoliosis in children with Duchenne muscular dystrophy: long-term follow-up. J Bone Joint Surg Am 2013;95:1057-1061.
42. Manzur AY, Kuntzer T, Pike M, Swan A. Glucocorticoid corticosteroids for Duchenne muscular dystrophy. Cochrane Database Syst Rev 2008:CD003725.
43. Henricson EK, Abresch RT, Cnaan A, et al. The cooperative international neuromuscular research group Duchenne natural history study: glucocorticoid treatment preserves clinically meaningful functional milestones and reduces rate of disease progression as measured by manual

muscle testing and other commonly used clinical trial outcome measures. Muscle Nerve 2013;48:55-67.
44. McAdam LC, Mayo AL, Alman BA, Biggar WD. The Canadian experience with long-term deflazacort treatment in Duchenne muscular dystrophy. Acta Myol 2012;31:16-20.
45. Biggar WD, Harris VA, Eliasoph L, Alman B. Long-term benefits of deflazacort treatment for boys with Duchenne muscular dystrophy in their second decade. Neuromuscul Disord 2006;16:249-255.
46. Dec GW. Steroid therapy effectively delays Duchenne's cardiomyopathy. J Am Coll Cardiol 2013;61:955-956.
47. Markham LW, Kinnett K, Wong BL, Woodrow Benson D, Cripe LH. Corticosteroid treatment retards development of ventricular dysfunction in Duchenne muscular dystrophy. Neuromuscul Disord 2008;18:365-370.
48. Finder JD, Birnkrant D, Carl J, et al. Respiratory care of the patient with Duchenne muscular dystrophy: ATS consensus statement. Am J Respir Crit Care Med 2004;170:456-465.
49. Cardiovascular health supervision for individuals affected by Duchenne or Becker muscular dystrophy. Pediatrics 2005;116:1569-1573.
50. Allen HD, Thrush PT, Hoffman TM, Flanigan KM, Mendell JR. Cardiac management in neuromuscular diseases. Phys Med Rehabil Clin N Am 2012;23:855-868.
51. Raman SV, Hor KN, Mazur W, et al. Eplerenone for early cardiomyopathy in Duchenne muscular dystrophy: a randomised, double-blind, placebo-controlled trial. The Lancet Neurology 2015;14:153-161.
52. Duboc D, Meune C, Lerebours G, Devaux JY, Vaksmann G, Becane HM. Effect of perindopril on the onset and progression of left ventricular dysfunction in Duchenne muscular dystrophy. J Am Coll Cardiol 2005;45:855-857.
53. Ogata H, Ishikawa Y, Minami R. Beneficial effects of beta-blockers and angiotensin-converting enzyme inhibitors in Duchenne muscular dystrophy. J Cardiol 2009;53:72-78.

54. Bianchi ML, Biggar D, Bushby K, Rogol AD, Rutter MM, Tseng B. Endocrine aspects of Duchenne muscular dystrophy. Neuromuscul Disord 2011;21:298-303.
55. Bianchi ML, Mazzanti A, Galbiati E, et al. Bone mineral density and bone metabolism in Duchenne muscular dystrophy. Osteoporos Int 2003;14:761-767.
56. Quinlivan R, Roper H, Davie M, Shaw NJ, McDonagh J, Bushby K. Report of a Muscular Dystrophy Campaign funded workshop Birmingham, UK, January 16th 2004. Osteoporosis in Duchenne muscular dystrophy; its prevalence, treatment and prevention. Neuromuscul Disord 2005;15:72-79.
57. Quinlivan R, Shaw N, Bushby K. 170th ENMC International Workshop: bone protection for corticosteroid treated Duchenne muscular dystrophy. 27-29 November 2009, Naarden, The Netherlands. Neuromuscul Disord 2010;20:761-769.
58. Rutter MM, Collins J, Rose SR, et al. Growth hormone treatment in boys with Duchenne muscular dystrophy and glucocorticoid-induced growth failure. Neuromuscul Disord 2012;22:1046-1056.
59. Ricotti V, Ridout DA, Muntoni F. Steroids in Duchenne muscular dystrophy. Neuromuscul Disord 2013;23:696-697.
60. Leon SH, Schuffler MD, Kettler M, Rohrmann CA. Chronic intestinal pseudoobstruction as a complication of Duchenne's muscular dystrophy. Gastroenterology 1986;90:455-459.
61. Dinan D, Levine MS, Gordon AR, Rubesin SE, Rombeau JL. Gastric wall weakening resulting in separate perforations in a patient with Duchenne's muscular dystrophy. AJR Am J Roentgenol 2003;181:807-808.
62. V. Ricotti EE, A. Emmanuel, J. Knowles, D. Walker, P. Giordano, A. Simonds, M. Hanna, and others. Recurrent pseudo-obstruction and sigmoid volvulus in Duchenne Muscular Dystrophy: A case report. Neuromuscular Disorders, ;22:p887-888.
63. Anziska Y, Sternberg A. Exercise in neuromuscular disease. Muscle Nerve 2013;48:3-20.

64. Green JM, Murton FE. Diagnosis of Duchenne muscular dystrophy: parents' experiences and satisfaction. Child Care Health Dev 1996;22:113-128.
65. Nereo NE, Fee RJ, Hinton VJ. Parental stress in mothers of boys with duchenne muscular dystrophy. J Pediatr Psychol 2003;28:473-484.
66. Felisari G, Martinelli Boneschi F, Bardoni A, et al. Loss of Dp140 dystrophin isoform and intellectual impairment in Duchenne dystrophy. Neurology 2000;55:559-564.
67. Daoud F, Angeard N, Demerre B, et al. Analysis of Dp71 contribution in the severity of mental retardation through comparison of Duchenne and Becker patients differing by mutation consequences on Dp71 expression. Hum Mol Genet 2009;18:3779-3794.
68. Lorusso ML, Civati F, Molteni M, Turconi AC, Bresolin N, D'Angelo MG. Specific profiles of neurocognitive and reading functions in a sample of 42 Italian boys with Duchenne Muscular Dystrophy. Child Neuropsychol 2013;19:350-369.
69. Wu JY, Kuban KC, Allred E, Shapiro F, Darras BT. Association of Duchenne muscular dystrophy with autism spectrum disorder. J Child Neurol 2005;20:790-795.
70. Hendriksen JG, Vles JS. Neuropsychiatric disorders in males with duchenne muscular dystrophy: frequency rate of attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder, and obsessive--compulsive disorder. J Child Neurol 2008;23:477-481.
71. Hinton VJ, Cyrulnik SE, Fee RJ, et al. Association of autistic spectrum disorders with dystrophinopathies. Pediatr Neurol 2009;41:339-346.
72. Pane M, Lombardo ME, Alfieri P, et al. Attention deficit hyperactivity disorder and cognitive function in Duchenne muscular dystrophy: phenotype-genotype correlation. J Pediatr 2012;161:705-709 e701.
73. Banihani R, Smile S, Yoon G, et al. Cognitive and Neurobehavioral Profile in Boys With Duchenne Muscular Dystrophy. J Child Neurol 2015.
74. Seto JT, Bengtsson NE, Chamberlain JS. Therapy of Genetic Disorders-Novel Therapies for Duchenne Muscular Dystrophy. Curr Pediatr Rep 2014;2:102-112.

75. Mercuri E, Muntoni F. Muscular dystrophies. Lancet 2013;381:845-860.
76. Kinali M, Arechavala-Gomeza V, Feng L, et al. Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. Lancet Neurol 2009;8:918-928.
77. van Deutekom JC, Janson AA, Ginjaar IB, et al. Local dystrophin restoration with antisense oligonucleotide PRO051. N Engl J Med 2007;357:2677-2686.
78. Goemans NM, Tulinius M, van den Akker JT, et al. Systemic administration of PRO051 in Duchenne's muscular dystrophy. N Engl J Med 2011;364:1513-1522.
79. Cirak S, Arechavala-Gomeza V, Guglieri M, et al. Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. Lancet 2011;378:595-605.
80. Mendell JR, Rodino-Klapac LR, Sahenk Z, et al. Eteplirsen for the treatment of Duchenne muscular dystrophy. Ann Neurol 2013;74:637-647.
81. Voit T, Topaloglu H, Straub V, et al. Safety and efficacy of drisapersen for the treatment of Duchenne muscular dystrophy (DEMAND II): an exploratory, randomised, placebo-controlled phase 2 study. The Lancet Neurology 2014;13:987-996.
82. Bushby K, Finkel R, Wong B, et al. Ataluren treatment of patients with nonsense mutation dystrophinopathy. Muscle Nerve 2014;50:477-487.
83. Duchenne G. De l'electrisation localisee. Paris, Balliere. 1868:595-616.
84. Conte GG, L. Scrofola del sistema muscolare. Annale Clinici dell'Ospedale degli Incurabili di Napoli 1952:66-79.
85. Meryon E. On granular and fatty degeneration of the voluntary muscles. . Medico-Chirurgical Trans 1952;35:73-74.
86. Maryon E. On granular and fatty degeneration of the voluntary muscles. Lancet 1951:588-589.
87. Clarke JG, WR. On a case of pseudo-hypertrophic muscular paralysi. Med-Chir Trans 1875:801-802.

88. Gowers W. Clinical lectures on pseudo-hypertrophic muscular paralysis. Lancet 1979:1-2, 37-49, 73-35, 113-116.
89. Okinaka S, Kumagai H, Ebashi S, et al. Serum creatine phosphokinase. Activity in progressive muscular dystrophy and neuromuscular diseases. Arch Neurol 1961;4:520-525.
90. Davies KE, Pearson PL, Harper PS, et al. Linkage analysis of two cloned DNA sequences flanking the Duchenne muscular dystrophy locus on the short arm of the human X chromosome. Nucleic Acids Res 1983;11:2303-2312.
91. Hoffman EP, Brown RH, Jr., Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 1987;51:919-928.
92. Hoffman EP, Knudson CM, Campbell KP, Kunkel LM. Subcellular fractionation of dystrophin to the triads of skeletal muscle. Nature 1987;330:754-758.
93. Miyatake M, Miike T, Zhao JE, Yoshioka K, Uchino M, Usuku G. Dystrophin: localization and presumed function. Muscle Nerve 1991;14:113-119.
94. Becker P, Kiener, F. A new X-Crhomosomal muscular Dystrophy. Arch Pshychiatr Nervenkr Z Gesamte Neurol Psychiatr 1995;4:427-448.
95. Drachman DB, Toyka KV, Myer E. Prednisone in Duchenne muscular dystrophy. Lancet 1974;2:1409-1412.
96. Siegel IM, Miller JE, Ray RD. Failure of corticosteroid in the treatment of Duchenne (pseudo-hypertrophic) muscular dystrophy. Report of a clinically matched three year double-blind study. IMJ Ill Med J 1974;145:32-33 passim.
97. Brooke MH, Fenichel GM, Griggs RC, et al. Clinical investigation of Duchenne muscular dystrophy. Interesting results in a trial of prednisone. Arch Neurol 1987;44:812-817.
98. Mendell JR, Province MA, Moxley RT, 3rd, et al. Clinical investigation of Duchenne muscular dystrophy. A methodology for therapeutic trials based on natural history controls. Arch Neurol 1987;44:808-811.

99. DeSilva S, Drachman DB, Mellits D, Kuncl RW. Prednisone treatment in Duchenne muscular dystrophy. Long-term benefit. Arch Neurol 1987;44:818-822.
100. Brooke MH, Fenichel GM, Griggs RC, et al. Duchenne muscular dystrophy: patterns of clinical progression and effects of supportive therapy. Neurology 1989;39:475-481.
101. Mendell JR, Moxley RT, Griggs RC, et al. Randomized, double-blind six-month trial of prednisone in Duchenne's muscular dystrophy. N Engl J Med 1989;320:1592-1597.
102. Fenichel GM, Mendell JR, Moxley RT, 3rd, et al. A comparison of daily and alternate-day prednisone therapy in the treatment of Duchenne muscular dystrophy. Arch Neurol 1991;48:575-579.
103. Griggs RC, Moxley RT, 3rd, Mendell JR, et al. Prednisone in Duchenne dystrophy. A randomized, controlled trial defining the time course and dose response. Clinical Investigation of Duchenne Dystrophy Group. Arch Neurol 1991;48:383-388.
104. Fenichel GM, Florence JM, Pestronk A, et al. Long-term benefit from prednisone therapy in Duchenne muscular dystrophy. Neurology 1991;41:1874-1877.
105. Dubowitz V. Prednisone in Duchenne dystrophy. Neuromuscul Disord 1991;1:161-163.
106. Sansome A, Royston P, Dubowitz V. Steroids in Duchenne muscular dystrophy; pilot study of a new low-dosage schedule. Neuromuscul Disord 1993;3:567-569.
107. Beenakker EA, Fock JM, Van Tol MJ, et al. Intermittent prednisone therapy in Duchenne muscular dystrophy: a randomized controlled trial. Arch Neurol 2005;62:128-132.
108. Kinali M, Mercuri E, Main M, Muntoni F, Dubowitz V. An effective, low-dosage, intermittent schedule of prednisolone in the long-term treatment of early cases of Duchenne dystrophy. Neuromuscul Disord 2002;12 Suppl 1:S169-174.

109. Dubowitz V, Kinali M, Main M, Mercuri E, Muntoni F. Remission of clinical signs in early duchenne muscular dystrophy on intermittent low-dosage prednisolone therapy. Eur J Paediatr Neurol 2002;6:153-159.
110. Connolly AM, Schierbecker J, Renna R, Florence J. High dose weekly oral prednisone improves strength in boys with Duchenne muscular dystrophy. Neuromuscul Disord 2002;12:917-925.
111. Bonifati MD, Ruzza G, Bonometto P, et al. A multicenter, double-blind, randomized trial of deflazacort versus prednisone in Duchenne muscular dystrophy. Muscle Nerve 2000;23:1344-1347.
112. Pradhan S, Ghosh D, Srivastava NK, et al. Prednisolone in Duchenne muscular dystrophy with imminent loss of ambulation. J Neurol 2006;253:1309-1316.
113. Mazzone E, Vasco G, Sormani MP, et al. Functional changes in Duchenne muscular dystrophy: A 12-month longitudinal cohort study. Neurology 2011;77:250-256.
114. Escolar DM, Hache LP, Clemens PR, et al. Randomized, blinded trial of weekend vs daily prednisone in Duchenne muscular dystrophy. Neurology 2011;77:444-452.
115. Moxley RT, 3rd, Pandya S. Weekend high-dosage prednisone: a new option for treatment of Duchenne muscular dystrophy. Neurology 2011;77:416-417.
116. Bello L, Gordish-Dressman H, Morgenroth LP, et al. Prednisone/prednisolone and deflazacort regimens in the CINRG Duchenne Natural History Study. Neurology 2015.
117. Pane M, Fanelli L, Mazzone ES, et al. Benefits of glucocorticoids in non-ambulant boys/men with Duchenne muscular dystrophy: A multicentric longitudinal study using the Performance of Upper Limb test. Neuromuscul Disord 2015;25:749-753.
118. McKay LI, DuBois DC, Sun YN, Almon RR, Jusko WJ. Corticosteroid effects in skeletal muscle: gene induction/receptor autoregulation. Muscle Nerve 1997;20:1318-1320.
119. Khan MA. Corticosteroid therapy in Duchenne muscular dystrophy. J Neurol Sci 1993;120:8-14.

120. Kissel JT, Burrow KL, Rammohan KW, Mendell JR. Mononuclear cell analysis of muscle biopsies in prednisone-treated and untreated Duchenne muscular dystrophy. CIDD Study Group. Neurology 1991;41:667-672.
121. Muntoni F, Fisher I, Morgan JE, Abraham D. Steroids in Duchenne muscular dystrophy: from clinical trials to genomic research. Neuromuscul Disord 2002;12 Suppl 1:S162-165.
122. Fisher I, Abraham D, Bouri K, Hoffman EP, Muntoni F, Morgan J. Prednisolone-induced changes in dystrophic skeletal muscle. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2005;19:834-836.
123. Center for Human and Clinical Genetics LUMC. Available at: http://www.dmd.nl.
124. Scott E, Eagle M, Mayhew A, et al. Development of a functional assessment scale for ambulatory boys with Duchenne muscular dystrophy. Physiother Res Int 2012;17:101-109.
125. Mazzone ES, Messina S, Vasco G, et al. Reliability of the North Star Ambulatory Assessment in a multicentric setting. Neuromuscul Disord 2009;19:458-461.
126. Mazzone E, Martinelli D, Berardinelli A, et al. North Star Ambulatory Assessment, 6-minute walk test and timed items in ambulant boys with Duchenne muscular dystrophy. Neuromuscul Disord 2010;20:712-716.
127. Mayhew A, Cano S, Scott E, Eagle M, Bushby K, Muntoni F. Moving towards meaningful measurement: Rasch analysis of the North Star Ambulatory Assessment in Duchenne muscular dystrophy. Dev Med Child Neurol 2011;53:535-542.
128. Hamilton L. Statistics with STATA, 8th Edition ed: Cengage Learning, Inc;, 2011.
129. Mazzone ES, Pane M, Sormani MP, et al. Correction: 24 Month Longitudinal Data in Ambulant Boys with Duchenne Muscular Dystrophy. PLoS One 2013;8.
130. White IR, Royston P, Wood AM. Multiple imputation using chained equations: Issues and guidance for practice. Stat Med 2011;30:377-399.

131. Cole TJ. Growth monitoring with the British 1990 growth reference. Arch Dis Child 1997;76:47-49.
132. StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP.
133. Emery AE. The muscular dystrophies. Lancet 2002;359:687-695.
134. McDonald CM, Han JJ, Mah JK, Carter GT. Corticosteroids and Duchenne muscular dystrophy: does earlier treatment really matter? Muscle Nerve 2012;45:777-779.
135. Merlini L, Gennari M, Malaspina E, et al. Early corticosteroid treatment in 4 Duchenne muscular dystrophy patients: 14-year follow-up. Muscle Nerve 2012;45:796-802.
136. Mayo AL, Craven BC, McAdam LC, Biggar WD. Bone health in boys with Duchenne Muscular Dystrophy on long-term daily deflazacort therapy. Neuromuscul Disord 2012.
137. King WM, Ruttencutter R, Nagaraja HN, et al. Orthopedic outcomes of long-term daily corticosteroid treatment in Duchenne muscular dystrophy. Neurology 2007;68:1607-1613.
138. Black N. Why we need observational studies to evaluate the effectiveness of health care. Bmj 1996;312:1215-1218.
139. Benson K, Hartz AJ. A comparison of observational studies and randomized, controlled trials. N Engl J Med 2000;342:1878-1886.
140. Vandenbroucke JP. When are observational studies as credible as randomised trials? Lancet 2004;363:1728-1731.
141. Avorn J. In defense of pharmacoepidemiology--embracing the yin and yang of drug research. N Engl J Med 2007;357:2219-2221.
142. Tannen RL, Weiner MG, Xie D. Use of primary care electronic medical record database in drug efficacy research on cardiovascular outcomes: comparison of database and randomised controlled trial findings. Bmj 2009;338:b81.
143. Bushby K, Griggs R. FOR-DMD; Finding the optimum regimen for DMD. An NIH funded trial of steroids. [online]. Available at: http://www.parentprojectmd.org/site/DocServer/7-8-11-Bushby.pdf?docID=11610.

144. Welch EM, Barton ER, Zhuo J, et al. PTC124 targets genetic disorders caused by nonsense mutations. Nature 2007;447:87-91.
145. Hirawat S, Welch EM, Elfring GL, et al. Safety, tolerability, and pharmacokinetics of PTC124, a nonaminoglycoside nonsense mutation suppressor, following single- and multiple-dose administration to healthy male and female adult volunteers. J Clin Pharmacol 2007;47:430-444.
146. Buyse GM, Goemans N, van den Hauwe M, Meier T. Effects of glucocorticoids and idebenone on respiratory function in patients with duchenne muscular dystrophy. Pediatr Pulmonol 2013;48:912-920.
147. Nelson MD, Rader F, Tang X, et al. PDE5 inhibition alleviates functional muscle ischemia in boys with Duchenne muscular dystrophy. Neurology 2014;82:2085-2091.
148. Tinsley JM, Fairclough RJ, Storer R, et al. Daily treatment with SMTC1100, a novel small molecule utrophin upregulator, dramatically reduces the dystrophic symptoms in the mdx mouse. PLoS One 2011;6:e19189.
149. Koo T, Okada T, Athanasopoulos T, Foster H, Takeda S, Dickson G. Long-term functional adeno-associated virus-microdystrophin expression in the dystrophic CXMDj dog. J Gene Med 2011;13:497-506.
150. Odom GL, Gregorevic P, Allen JM, Chamberlain JS. Gene therapy of mdx mice with large truncated dystrophins generated by recombination using rAAV6. Mol Ther 2011;19:36-45.
151. Gregorevic P, Blankinship MJ, Allen JM, Chamberlain JS. Systemic microdystrophin gene delivery improves skeletal muscle structure and function in old dystrophic mdx mice. Mol Ther 2008;16:657-664.
152. Vulin A, Barthelemy I, Goyenvalle A, et al. Muscle function recovery in golden retriever muscular dystrophy after AAV1-U7 exon skipping. Mol Ther 2012;20:2120-2133.
153. Incitti T, De Angelis FG, Cazzella V, et al. Exon skipping and duchenne muscular dystrophy therapy: selection of the most active U1 snRNA antisense able to induce dystrophin exon 51 skipping. Mol Ther 2010;18:1675-1682.

154. Le Guiner C, Montus M, Servais L, et al. Forelimb treatment in a large cohort of dystrophic dogs supports delivery of a recombinant AAV for exon skipping in Duchenne patients. Mol Ther 2014;22:1923-1935.
155. Bushby KM, Gardner-Medwin D. The clinical, genetic and dystrophin characteristics of Becker muscular dystrophy. I. Natural history. J Neurol 1993;240:98-104.
156. Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics 1988;2:90-95.
157. Arechavala-Gomeza V, Anthony K, Morgan J, Muntoni F. Antisense oligonucleotide-mediated exon skipping for Duchenne muscular dystrophy: progress and challenges. Curr Gene Ther 2012;12:152-160.
158. Finkel RS, Flanigan KM, Wong B, et al. Phase 2a study of ataluren-mediated dystrophin production in patients with nonsense mutation duchenne muscular dystrophy. PLoS One 2013;8:e81302.
159. Mazzone ES, Pane M, Sormani MP, et al. 24 month longitudinal data in ambulant boys with Duchenne muscular dystrophy. PLoS One 2013;8:e52512.
160. McDonald CM, Henricson EK, Abresch RT, et al. The cooperative international neuromuscular research group Duchenne natural history study--a longitudinal investigation in the era of glucocorticoid therapy: design of protocol and the methods used. Muscle Nerve 2013;48:32-54.
161. McDonald CM, Henricson EK, Abresch RT, et al. The 6-minute walk test and other endpoints in Duchenne muscular dystrophy: longitudinal natural history observations over 48 weeks from a multicenter study. Muscle Nerve 2013;48:343-356.
162. Anthony K, Arechavala-Gomeza V, Ricotti V, et al. Biochemical Characterization of Patients With In-Frame or Out-of-Frame DMD Deletions Pertinent to Exon 44 or 45 Skipping. JAMA Neurol 2014;71:32-40.
163. van den Bergen JC, Wokke BH, Janson AA, et al. Dystrophin levels and clinical severity in Becker muscular dystrophy patients. J Neurol Neurosurg Psychiatry 2013.

164. McDonald CM, Henricson EK, Han JJ, et al. The 6-minute walk test as a new outcome measure in Duchenne muscular dystrophy. Muscle Nerve 2010;41:500-510.
165. Mayhew AG, Cano SJ, Scott E, et al. Detecting meaningful change using the North Star Ambulatory Assessment in Duchenne muscular dystrophy. Dev Med Child Neurol 2013;55:1046-1052.
166. Henricson E, Abresch R, Han JJ, et al. Percent-predicted 6-minute walk distance in duchenne muscular dystrophy to account for maturational influences. PLoS Curr 2012;4:RRN1297.
167. Cirak S, Feng L, Anthony K, et al. Restoration of the dystrophin-associated glycoprotein complex after exon skipping therapy in Duchenne muscular dystrophy. Mol Ther 2012;20:462-467.
168. Pane M, Mazzone ES, Sormani MP, et al. 6 minute walk test in Duchenne MD patients with different mutations: 12 month changes. PLoS One 2014;9:e83400.
169. Servais L. Non Ambulant patients with deletion treatable by exon skipping 53 present a more severe phenotype than the general Duchenne population. In: Neuromuscular Disorder, Elsevier 2013: 843.
170. Anthony K, Cirak S, Torelli S, et al. Dystrophin quantification and clinical correlations in Becker muscular dystrophy: implications for clinical trials. Brain 2011;134:3547-3559.
171. Fletcher S, Adkin CF, Meloni P, et al. Targeted exon skipping to address "leaky" mutations in the dystrophin gene. Mol Ther Nucleic Acids 2012;1:e48.
172. Pegoraro E, Hoffman EP, Piva L, et al. SPP1 genotype is a determinant of disease severity in Duchenne muscular dystrophy. Neurology 2011;76:219-226.
173. Bello L, Piva L, Barp A, et al. Importance of SPP1 genotype as a covariate in clinical trials in Duchenne muscular dystrophy. Neurology 2012;79:159-162.
174. Flanigan KM, Ceco E, Lamar KM, et al. LTBP4 genotype predicts age of ambulatory loss in duchenne muscular dystrophy. Ann Neurol 2012.

175. van den Bergen JC, Hiller M, Bohringer S, et al. Validation of genetic modifiers for Duchenne muscular dystrophy: a multicentre study assessing SPP1 and LTBP4 variants. J Neurol Neurosurg Psychiatry 2014.
176. Walton JN, Nattrass FJ. On the classification, natural history and treatment of the myopathies. Brain 1954;77:169-231.
177. Schoelly ML, Fraser AW. Emotional reactions in muscular dystrophy. Am J Phys Med 1955;34:119-123.
178. Morrow RS, Cohen J. The psychosocial factors in muscular dystrophy. J Child Psychiatry 1954;3:70-80.
179. Truitt CJ. Personal and social adjustments of children with muscular dystrophy. Am J Phys Med 1955;34:124-128.
180. Allen JE, Rodgin DW. Mental retardation in association with progressive dystrophy. Am J Dis Child 1960;100:208-211.
181. Worden DK, Vignos PJ, Jr. Intellectual function in childhood progressive muscular dystrophy. Pediatrics 1962;29:968-977.
182. Dubowitz V. Intellectual Impairment in Muscular Dystrophy. Arch Dis Child 1965;40:296-301.
183. Zellweger H, Niedermeyer E. Central nervous system manifestations in childhood muscular dystrophy (CMD). I. Psychometric and electroencephalographic findings. Ann Paediatr 1965;205:25-42.
184. Zellweger H, Hanson JW. Psychometric studies in muscular dystrophy type 3a (Duchenne). Dev Med Child Neurol 1967;9:576-581.
185. Prosser EJ, Murphy EG, Thompson MW. Intelligence and the gene for Duchenne muscular dystrophy. Arch Dis Child 1969;44:221-230.
186. Leibowitz D, Dubowitz V. Intellect and behaviour in Duchenne muscular dystrophy. Dev Med Child Neurol 1981;23:577-590.
187. Ervasti JM. Dystrophin, its interactions with other proteins, and implications for muscular dystrophy. Biochim Biophys Acta 2007;1772:108-117.
188. Le Rumeur E, Winder SJ, Hubert JF. Dystrophin: more than just the sum of its parts. Biochim Biophys Acta 2010;1804:1713-1722.
189. Anderson JL, Head SI, Rae C, Morley JW. Brain function in Duchenne muscular dystrophy. Brain 2002;125:4-13.

190. Pillers DA, Weleber RG, Green DG, et al. Effects of dystrophin isoforms on signal transduction through neural retina: genotype-phenotype analysis of duchenne muscular dystrophy mouse mutants. Mol Genet Metab 1999;66:100-110.
191. Pillers DA, Fitzgerald KM, Duncan NM, et al. Duchenne/Becker muscular dystrophy: correlation of phenotype by electroretinography with sites of dystrophin mutations. Hum Genet 1999;105:2-9.
192. Rutter M. Incidence of autism spectrum disorders: changes over time and their meaning. Acta Paediatr 2005;94:2-15.
193. Willcutt EG. The prevalence of DSM-IV attention-deficit/hyperactivity disorder: a meta-analytic review. Neurotherapeutics 2012;9:490-499.
194. General USPHSOotS. Mental Health: A Report of the Surgeon Genera, 1999.
195. Sarvet B. Childhood obsessive-compulsive disorder. Pediatr Rev 2013;34:19-27; quiz 28.
196. Skuse DH, Mandy WP, Scourfield J. Measuring autistic traits: heritability, reliability and validity of the Social and Communication Disorders Checklist. Br J Psychiatry 2005;187:568-572.
197. Skuse DH, Mandy W, Steer C, et al. Social communication competence and functional adaptation in a general population of children: preliminary evidence for sex-by-verbal IQ differential risk. J Am Acad Child Adolesc Psychiatry 2009;48:128-137.
198. Wechsler D. Weschler Intelligence Scale for Children-fourth edition (WISC-IV). London: Pearsons Assessment, 2004.
199. Raiford SE, Weiss, L.G., Rolfhus, E., Coalson, D. WISC-IV General Ability Index (WISC-IVTechnical Report No. 4), 2005.
200. Wechsler D. Wechsler Preschool and Primary Scale of Intelligence™, Third ed: Pearsons, 2002.
201. Raven J. Raven's Coloured Progressive Matrices: Pearson, 1995.
202. Esther Strauss EMSS, Otfried Spreen. A Compendium of Neuropsychological Tests: Administration, Norms, and Commentary Oxford University Press, New York, 2006.

203. American Psychiatric Association. APA. Diagnostic and statistical manual of mental disorders : DSM-IV-TR., 2000.
204. Santosh PJ, Mandy WP, Puura K, Kaartinen M, Warrington R, Skuse DH. The construction and validation of a short form of the developmental, diagnostic and dimensional interview. Eur Child Adolesc Psychiatry 2009;18:521-524.
205. Conners CK, Sitarenios G, Parker JD, Epstein JN. The revised Conners' Parent Rating Scale (CPRS-R): factor structure, reliability, and criterion validity. J Abnorm Child Psychol 1998;26:257-268.
206. Achenbach T. Manual for the Child Behavior Checklist/4 - 18 and 1991 Profile.: Burlington, VT. University of Vermont Department of Psychiatry, 1991.
207. Field A. Discovering Statistics using SPSS, Third Edition ed, 2011.
208. Skuse D, Lawrence K, Tang J. Measuring social-cognitive functions in children with somatotropic axis dysfunction. Horm Res 2005;64 Suppl 3:73-82.
209. Fee RJ, Hinton VJ. Resilience in children diagnosed with a chronic neuromuscular disorder. J Dev Behav Pediatr 2011;32:644-650.
210. Cotton S, Voudouris NJ, Greenwood KM. Intelligence and Duchenne muscular dystrophy: full-scale, verbal, and performance intelligence quotients. Dev Med Child Neurol 2001;43:497-501.
211. Hinton VJ, De Vivo DC, Nereo NE, Goldstein E, Stern Y. Selective deficits in verbal working memory associated with a known genetic etiology: the neuropsychological profile of duchenne muscular dystrophy. J Int Neuropsychol Soc 2001;7:45-54.
212. Simonoff E, Pickles A, Charman T, Chandler S, Loucas T, Baird G. Psychiatric disorders in children with autism spectrum disorders: prevalence, comorbidity, and associated factors in a population-derived sample. J Am Acad Child Adolesc Psychiatry 2008;47:921-929.
213. Taylor B, Jick H, Maclaughlin D. Prevalence and incidence rates of autism in the UK: time trend from 2004-2010 in children aged 8 years. BMJ Open 2013;3:e003219.

214. Polanczyk G, de Lima MS, Horta BL, Biederman J, Rohde LA. The worldwide prevalence of ADHD: a systematic review and metaregression analysis. Am J Psychiatry 2007;164:942-948.
215. Hiscock H, Bayer JK, Lycett K, et al. Preventing mental health problems in children: the Families in Mind population-based cluster randomised controlled trial. BMC Public Health 2012;12:420.
216. Hendriksen JG, Poysky JT, Schrans DG, Schouten EG, Aldenkamp AP, Vles JS. Psychosocial adjustment in males with Duchenne muscular dystrophy: psychometric properties and clinical utility of a parent-report questionnaire. J Pediatr Psychol 2009;34:69-78.
217. Darke J, Bushby K, Le Couteur A, McConachie H. Survey of behaviour problems in children with neuromuscular diseases. Eur J Paediatr Neurol 2006;10:129-134.
218. Taylor PJ, Betts GA, Maroulis S, et al. Dystrophin gene mutation location and the risk of cognitive impairment in Duchenne muscular dystrophy. PLoS One 2010;5:e8803.
219. Sekiguchi M, Zushida K, Yoshida M, et al. A deficit of brain dystrophin impairs specific amygdala GABAergic transmission and enhances defensive behaviour in mice. Brain 2009;132:124-135.
220. Nicchia GP, Rossi A, Nudel U, Svelto M, Frigeri A. Dystrophin-dependent and -independent AQP4 pools are expressed in the mouse brain. Glia 2008;56:869-876.
221. Haenggi T, Soontornmalai A, Schaub MC, Fritschy JM. The role of utrophin and Dp71 for assembly of different dystrophin-associated protein complexes (DPCs) in the choroid plexus and microvasculature of the brain. Neuroscience 2004;129:403-413.
222. Doorenweerd N, Straathof CS, Dumas EM, et al. Reduced cerebral gray matter and altered white matter in boys with Duchenne muscular dystrophy. Ann Neurol 2014.
223. Tracey I, Scott RB, Thompson CH, et al. Brain abnormalities in Duchenne muscular dystrophy: phosphorus-31 magnetic resonance spectroscopy and neuropsychological study. Lancet 1995;345:1260-1264.

224. Goodnough CL, Gao Y, Li X, et al. Lack of dystrophin results in abnormal cerebral diffusion and perfusion in vivo. Neuroimage 2014;102P2:809-816.
225. Goyenvalle A, Griffith G, Babbs A, et al. Functional correction in mouse models of muscular dystrophy using exon-skipping tricyclo-DNA oligomers. Nat Med 2015.
226. Forrester J DA, McMenamin P, Lee W. The Eye. Basic Sciences in Practise: Saunders, 2005.
227. Forrester JV DA, McMenamin PG, Lee WR. The Eye, Basic Sciences in Practise, Second ed: Saunders, 2002.
228. Hoon M, Okawa H, Della Santina L, Wong RO. Functional architecture of the retina: development and disease. Prog Retin Eye Res 2014;42:44-84.
229. Reichenbach A, Bringmann A. New functions of Muller cells. Glia 2013;61:651-678.
230. Ueda H, Baba T, Terada N, Kato Y, Tsukahara S, Ohno S. Dystrophin in rod spherules; submembranous dense regions facing bipolar cell processes. Histochem Cell Biol 1997;108:243-248.
231. Dalloz C, Sarig R, Fort P, et al. Targeted inactivation of dystrophin gene product Dp71: phenotypic impact in mouse retina. Hum Mol Genet 2003;12:1543-1554.
232. Tadayoni R, Rendon A, Soria-Jasso LE, Cisneros B. Dystrophin Dp71: the smallest but multifunctional product of the Duchenne muscular dystrophy gene. Mol Neurobiol 2012;45:43-60.
233. Wersinger E, Bordais A, Schwab Y, et al. Reevaluation of dystrophin localization in the mouse retina. Invest Ophthalmol Vis Sci 2011;52:7901-7908.
234. Rodius F, Claudepierre T, Rosas-Vargas H, et al. Dystrophins in developing retina: Dp260 expression correlates with synaptic maturation. Neuroreport 1997;8:2383-2387.
235. Howard PL, Dally GY, Wong MH, et al. Localization of dystrophin isoform Dp71 to the inner limiting membrane of the retina suggests a unique functional contribution of Dp71 in the retina. Hum Mol Genet 1998;7:1385-1391.

236. Pillers DA. Dystrophin and the retina. Mol Genet Metab 1999;68:304-309.
237. Cia D, Simonutti M, Fort PE, Doly M, Rendon A. Slight alteration of the electroretinogram in mice lacking dystrophin dp71. Ophthalmic Res 2014;51:196-203.
238. Kameya S, Araki E, Katsuki M, et al. Dp260 disrupted mice revealed prolonged implicit time of the b-wave in ERG and loss of accumulation of beta-dystroglycan in the outer plexiform layer of the retina. Hum Mol Genet 1997;6:2195-2203.
239. Pillers DA, Weleber RG, Woodward WR, Green DG, Chapman VM, Ray PN. mdxCv3 mouse is a model for electroretinography of Duchenne/Becker muscular dystrophy. Invest Ophthalmol Vis Sci 1995;36:462-466.
240. Barboni MT, Nagy BV, de Araujo Moura AL, et al. ON and OFF electroretinography and contrast sensitivity in Duchenne muscular dystrophy. Invest Ophthalmol Vis Sci 2013;54:3195-3204.
241. Costa MF, Oliveira AG, Feitosa-Santana C, Zatz M, Ventura DF. Red-green color vision impairment in Duchenne muscular dystrophy. Am J Hum Genet 2007;80:1064-1075.
242. Marmor MF, Fulton AB, Holder GE, Miyake Y, Brigell M, Bach M. ISCEV Standard for full-field clinical electroretinography (2008 update). Doc Ophthalmol 2009;118:69-77.
243. Ricotti V, Ridout DA, Scott E, et al. Long-term benefits and adverse effects of intermittent versus daily glucocorticoids in boys with Duchenne muscular dystrophy. J Neurol Neurosurg Psychiatry 2012.
244. McCulloch DL, Marmor MF, Brigell MG, et al. ISCEV Standard for full-field clinical electroretinography (2015 update). Doc Ophthalmol 2015;130:1-12.
245. Kriss A. Skin ERGs: their effectiveness in paediatric visual assessment, confounding factors, and comparison with ERGs recorded using various types of corneal electrode. Int J Psychophysiol 1994;16:137-146.
246. Thompson DA LA. Visual Electrophysiology: how can it help you and your patient. : Hoyt and Taylor 2012.

247. Prifitera A WL, Saklofske DH. The WISC-III in context: New York, Academic Press, 1998.
248. GraphPad Prism [computer program]. Version 5 2005.
249. Robson JG, Frishman LJ. Dissecting the dark-adapted electroretinogram. Doc Ophthalmol 1998;95:187-215.
250. Thompson DA, Feather S, Stanescu HC, et al. Altered electroretinograms in patients with KCNJ10 mutations and EAST syndrome. J Physiol 2011;589:1681-1689.
251. Girlanda P, Quartarone A, Buceti R, et al. Extra-muscle involvement in dystrophinopathies: an electroretinography and evoked potential study. J Neurol Sci 1997;146:127-132.
252. Lidov HG, Selig S, Kunkel LM. Dp140: a novel 140 kDa CNS transcript from the dystrophin locus. Hum Mol Genet 1995;4:329-335.
253. Dallerac G, Perronnet C, Chagneau C, et al. Rescue of a dystrophin-like protein by exon skipping normalizes synaptic plasticity in the hippocampus of the mdx mouse. Neurobiol Dis 2011;43:635-641.
254. Kersten HM, Roxburgh RH, Danesh-Meyer HV. Ophthalmic manifestations of inherited neurodegenerative disorders. Nat Rev Neurol 2014;10:349-362.
255. Heckmatt JZ, Dubowitz V, Leeman S. Detection of pathological change in dystrophic muscle with B-scan ultrasound imaging. Lancet 1980;1:1389-1390.
256. Mercuri E, Pichiecchio A, Allsop J, Messina S, Pane M, Muntoni F. Muscle MRI in inherited neuromuscular disorders: past, present, and future. J Magn Reson Imaging 2007;25:433-440.
257. Straub V, Carlier PG, Mercuri E. TREAT-NMD workshop: pattern recognition in genetic muscle diseases using muscle MRI: 25-26 February 2011, Rome, Italy. Neuromuscul Disord 2012;22 Suppl 2:S42-53.
258. Hollingsworth KG, de Sousa PL, Straub V, Carlier PG. Towards harmonization of protocols for MRI outcome measures in skeletal muscle studies: consensus recommendations from two TREAT-NMD NMR workshops, 2 May 2010, Stockholm, Sweden, 1-2 October 2009, Paris, France. Neuromuscul Disord 2012;22 Suppl 2:S54-67.

259. Matsumura K, Nakano I, Fukuda N, Ikehira H, Tateno Y, Aoki Y. Proton spin-lattice relaxation time of Duchenne dystrophy skeletal muscle by magnetic resonance imaging. Muscle Nerve 1988;11:97-102.
260. Matsumura K, Nakano I, Fukuda N, Ikehira H, Tateno Y, Aoki Y. Duchenne muscular dystrophy carriers. Proton spin-lattice relaxation times of skeletal muscles on magnetic resonance imaging. Neuroradiology 1989;31:373-376.
261. Huang Y, Majumdar S, Genant HK, et al. Quantitative MR relaxometry study of muscle composition and function in Duchenne muscular dystrophy. J Magn Reson Imaging 1994;4:59-64.
262. Kinali M, Arechavala-Gomeza V, Cirak S, et al. Muscle histology vs MRI in Duchenne muscular dystrophy. Neurology 2011;76:346-353.
263. Garrood P, Hollingsworth KG, Eagle M, et al. MR imaging in Duchenne muscular dystrophy: quantification of T1-weighted signal, contrast uptake, and the effects of exercise. J Magn Reson Imaging 2009;30:1130-1138.
264. Mathur S, Lott DJ, Senesac C, et al. Age-related differences in lower-limb muscle cross-sectional area and torque production in boys with Duchenne muscular dystrophy. Arch Phys Med Rehabil 2010;91:1051-1058.
265. Kim HK, Laor T, Horn PS, Racadio JM, Wong B, Dardzinski BJ. T2 mapping in Duchenne muscular dystrophy: distribution of disease activity and correlation with clinical assessments. Radiology 2010;255:899-908.
266. Marden FA, Connolly AM, Siegel MJ, Rubin DA. Compositional analysis of muscle in boys with Duchenne muscular dystrophy using MR imaging. Skeletal Radiol 2005;34:140-148.
267. Hollingsworth KG, Garrood P, Eagle M, Bushby K, Straub V. Magnetic resonance imaging in Duchenne muscular dystrophy: longitudinal assessment of natural history over 18 months. Muscle Nerve 2013;48:586-588.
268. Forbes SC, Willcocks RJ, Triplett WT, et al. Magnetic resonance imaging and spectroscopy assessment of lower extremity skeletal muscles in boys with Duchenne muscular dystrophy: a multicenter cross sectional study. PLoS One 2014;9:e106435.

269. Wren TA, Bluml S, Tseng-Ong L, Gilsanz V. Three-point technique of fat quantification of muscle tissue as a marker of disease progression in Duchenne muscular dystrophy: preliminary study. AJR Am J Roentgenol 2008;190:W8-12.
270. Wokke BH, van den Bergen JC, Versluis MJ, et al. Quantitative MRI and strength measurements in the assessment of muscle quality in Duchenne muscular dystrophy. Neuromuscul Disord 2014;24:409-416.
271. Kan HE, Klomp DW, Wong CS, et al. In vivo 31P MRS detection of an alkaline inorganic phosphate pool with short T1 in human resting skeletal muscle. NMR Biomed 2010;23:995-1000.
272. Torriani M, Townsend E, Thomas BJ, Bredella MA, Ghomi RH, Tseng BS. Lower leg muscle involvement in Duchenne muscular dystrophy: an MR imaging and spectroscopy study. Skeletal Radiol 2012;41:437-445.
273. Willcocks RJ, Arpan IA, Forbes SC, et al. Longitudinal measurements of MRI-T2 in boys with Duchenne muscular dystrophy: effects of age and disease progression. Neuromuscul Disord 2014;24:393-401.
274. Kim HK, Merrow AC, Shiraj S, Wong BL, Horn PS, Laor T. Analysis of fatty infiltration and inflammation of the pelvic and thigh muscles in boys with Duchenne muscular dystrophy (DMD): grading of disease involvement on MR imaging and correlation with clinical assessments. Pediatr Radiol 2013;43:1327-1335.
275. Kim HK, Serai S, Lindquist D, et al. Quantitative Skeletal Muscle MRI: Part 2, MR Spectroscopy and T2 Relaxation Time Mapping-Comparison Between Boys With Duchenne Muscular Dystrophy and Healthy Boys. AJR Am J Roentgenol 2015;205:W216-223.
276. Forbes SC, Walter GA, Rooney WD, et al. Skeletal muscles of ambulant children with Duchenne muscular dystrophy: validation of multicenter study of evaluation with MR imaging and MR spectroscopy. Radiology 2013;269:198-207.
277. Dixon WT. Simple proton spectroscopic imaging. Radiology 1984;153:189-194.
278. Glover GH. Multipoint Dixon technique for water and fat proton and susceptibility imaging. J Magn Reson Imaging 1991;1:521-530.

279. Glover GH, Schneider E. Three-point Dixon technique for true water/fat decomposition with B0 inhomogeneity correction. Magn Reson Med 1991;18:371-383.
280. Brooke MH, Griggs RC, Mendell JR, Fenichel GM, Shumate JB, Pellegrino RJ. Clinical trial in Duchenne dystrophy. I. The design of the protocol. Muscle Nerve 1981;4:186-197.
281. Edwards RH, Dawson MJ, Wilkie DR, Gordon RE, Shaw D. Clinical use of nuclear magnetic resonance in the investigation of myopathy. Lancet 1982;1:725-731.
282. Newman RJ, Bore PJ, Chan L, et al. Nuclear magnetic resonance studies of forearm muscle in Duchenne dystrophy. Br Med J (Clin Res Ed) 1982;284:1072-1074.
283. Wary C, Naulet T, Thibaud JL, Monnet A, Blot S, Carlier PG. Splitting of Pi and other (3)(1)P NMR anomalies of skeletal muscle metabolites in canine muscular dystrophy. NMR Biomed 2012;25:1160-1169.
284. Latroche C, Matot B, Martins-Bach A, et al. Structural and Functional Alterations of Skeletal Muscle Microvasculature in Dystrophin-Deficient mdx Mice. Am J Pathol 2015.
285. Wary C, Azzabou N, Giraudeau C, et al. Quantitative NMRI and NMRS identify augmented disease progression after loss of ambulation in forearms of boys with Duchenne muscular dystrophy. NMR Biomed 2015;28:1150-1162.
286. Arpan I, Willcocks RJ, Forbes SC, et al. Examination of effects of corticosteroids on skeletal muscles of boys with DMD using MRI and MRS. Neurology 2014;83:974-980.
287. Bishop C, Newbould R, Janiczek R, Campion G. Magnetic Resonance Imaging Assessments of two doses of Drisapersen in the Treatment of Ambulant Boys with Duchenne Muscular Dystrophy. In: Neurology, ed., 2015: Vol. 84 no. 14 Supplement P87.059.
288. Beekman C, Sipkens JA, Testerink J, et al. A sensitive, reproducible and objective immunofluorescence analysis method of dystrophin in individual fibers in samples from patients with duchenne muscular dystrophy. PLoS One 2014;9:e107494.

289. Alfano LL, LP Berry, KM Yin, H Dvorchik, Flanigan, KM Cripe, L Mendell, JR. Pilot Study evaluating motivation on the performance of times walking in boys with Duchenne muscular dystrophy. Neuromuscul Disord 2014;24:860.
290. Yushkevich PA, Piven J, Hazlett HC, et al. User-guided 3D active contour segmentation of anatomical structures: significantly improved efficiency and reliability. Neuroimage 2006;31:1116-1128.
291. Mayhew A, Mazzone ES, Eagle M, et al. Development of the Performance of the Upper Limb module for Duchenne muscular dystrophy. Dev Med Child Neurol 2013;55:1038-1045.
292. Pane M, Mazzone ES, Fanelli L, et al. Reliability of the Performance of Upper Limb assessment in Duchenne muscular dystrophy. Neuromuscul Disord 2014;24:201-206.
293. Servais L, Deconinck N, Moraux A, et al. Innovative methods to assess upper limb strength and function in non-ambulant Duchenne patients. Neuromuscul Disord 2013;23:139-148.
294. Seferian AM, Moraux A, Annoussamy M, et al. Upper limb strength and function changes during a one-year follow-up in non-ambulant patients with Duchenne Muscular Dystrophy: an observational multicenter trial. PLoS One 2015;10:e0113999.
295. Steffensen B, Hyde S, Lyager S, Mattsson E. Validity of the EK scale: a functional assessment of non-ambulatory individuals with Duchenne muscular dystrophy or spinal muscular atrophy. Physiother Res Int 2001;6:119-134.
296. Wokke BH, van den Bergen JC, Hooijmans MT, Verschuuren JJ, Niks EH, Kan HE. T2 relaxation times are increased in skeletal muscle of DMD but not BMD patients. Muscle Nerve 2015.
297. Li W, Zheng Y, Zhang W, Wang Z, Xiao J, Yuan Y. Progression and variation of fatty infiltration of the thigh muscles in Duchenne muscular dystrophy, a muscle magnetic resonance imaging study. Neuromuscul Disord 2015;25:375-380.

298. Bonati U, Hafner P, Schadelin S, et al. Quantitative muscle MRI: A powerful surrogate outcome measure in Duchenne muscular dystrophy. Neuromuscul Disord 2015;25:679-685.
299. Vohra RS, Lott D, Mathur S, et al. Magnetic Resonance Assessment of Hypertrophic and Pseudo-Hypertrophic Changes in Lower Leg Muscles of Boys with Duchenne Muscular Dystrophy and Their Relationship to Functional Measurements. PLoS One 2015;10:e0128915.
300. Gaeta M, Messina S, Mileto A, et al. Muscle fat-fraction and mapping in Duchenne muscular dystrophy: evaluation of disease distribution and correlation with clinical assessments. Preliminary experience. Skeletal Radiol 2012;41:955-961.
301. Akima H, Lott D, Senesac C, et al. Relationships of thigh muscle contractile and non-contractile tissue with function, strength, and age in boys with Duchenne muscular dystrophy. Neuromuscul Disord 2012;22:16-25.
302. Kovanlikaya A, Guclu C, Desai C, Becerra R, Gilsanz V. Fat quantification using three-point dixon technique: in vitro validation. Acad Radiol 2005;12:636-639.
303. SKIP-NMD. Available at: http://www.skip-nmd.eu/project. Accessed October 2015.
304. Bushby K, Connor E. Clinical outcome measures for trials in Duchenne muscular dystrophy: report from International Working Group meetings. Clin Investig (Lond) 2011;1:1217-1235.
305. Ricotti V, Ridout DA, Pane M, et al. The NorthStar Ambulatory Assessment in Duchenne muscular dystrophy: considerations for the design of clinical trials. J Neurol Neurosurg Psychiatry 2015.
306. Duboc D, Meune C, Pierre B, et al. Perindopril preventive treatment on mortality in Duchenne muscular dystrophy: 10 years' follow-up. Am Heart J 2007;154:596-602.
307. Cohn RD, van Erp C, Habashi JP, et al. Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states. Nat Med 2007;13:204-210.

308. Goyenvalle A, Griffith G, Babbs A, et al. Functional correction in mouse models of muscular dystrophy using exon-skipping tricyclo-DNA oligomers. Nat Med 2015;21:270-275.
309. Betts C, Saleh AF, Arzumanov AA, et al. Pip6-PMO, A New Generation of Peptide-oligonucleotide Conjugates With Improved Cardiac Exon Skipping Activity for DMD Treatment. Mol Ther Nucleic Acids 2012;1:e38.
310. Hinton VJ, Fee RJ, Goldstein EM, De Vivo DC. Verbal and memory skills in males with Duchenne muscular dystrophy. Dev Med Child Neurol 2007;49:123-128.
311. Ricotti V, Mandy WP, Scoto M, et al. Neurodevelopmental, emotional, and behavioural problems in Duchenne muscular dystrophy in relation to underlying dystrophin gene mutations. Dev Med Child Neurol 2015.
312. Taylor LE, Kaminoh YJ, Rodesch CK, Flanigan KM. Quantification of dystrophin immunofluorescence in dystrophinopathy muscle specimens. Neuropathol Appl Neurobiol 2012;38:591-601.
313. Arechavala-Gomeza V, Kinali M, Feng L, et al. Immunohistological intensity measurements as a tool to assess sarcolemma-associated protein expression. Neuropathol Appl Neurobiol 2010;36:265-274.
314. Anthony K, Feng L, Arechavala-Gomeza V, et al. Exon skipping quantification by quantitative reverse-transcription polymerase chain reaction in duchenne muscular dystrophy patients treated with the antisense oligomer eteplirsen. Hum Gene Ther Methods 2012;23:336-345.
315. Anthony K, Arechavala-Gomeza V, Taylor LE, et al. Dystrophin quantification: Biological and translational research implications. Neurology 2014;83:2062-2069.
316. van den Bergen JC, Wokke BH, Janson AA, et al. Dystrophin levels and clinical severity in Becker muscular dystrophy patients. J Neurol Neurosurg Psychiatry 2014;85:747-753.
317. Daftary AS, Crisanti M, Kalra M, Wong B, Amin R. Effect of long-term steroids on cough efficiency and respiratory muscle strength in patients with Duchenne muscular dystrophy. Pediatrics 2007;119:e320-324.

318. Bello L, Kesari A, Gordish-Dressman H, et al. Genetic modifiers of ambulation in the Cooperative International Neuromuscular Research Group Duchenne Natural History Study. Ann Neurol 2015;77:684-696.
319. Rouillon J, Poupiot J, Zocevic A, et al. Serum proteomic profiling reveals fragments of MYOM3 as potential biomarkers for monitoring the outcome of therapeutic interventions in muscular dystrophies. Hum Mol Genet 2015;24:4916-4932.
320. Nadarajah VD, van Putten M, Chaouch A, et al. Serum matrix metalloproteinase-9 (MMP-9) as a biomarker for monitoring disease progression in Duchenne muscular dystrophy (DMD). Neuromuscul Disord 2011;21:569-578.
321. Ricotti V, Jagle H, Theodorou M, Moore AT, Muntoni F, Thompson DA. Ocular and neurodevelopmental features of Duchenne muscular dystrophy: a signature of dystrophin function in the central nervous system. Eur J Hum Genet 2015.
322. Bemelmans AP, Duque S, Riviere C, et al. A single intravenous AAV9 injection mediates bilateral gene transfer to the adult mouse retina. PLoS One 2013;8:e61618.

Appendix

Publications originated from the work of this thesis:

Ricotti V, Ridout D, Scott E, Quinlivan R, Robb S, Manzur AY, Muntoni F, on behalf of NorthStar Clinical Network.
Long term benefits and adverse effects of intermittent versus daily glucocorticoids in boys with Duchenne Muscular Dystrophy. J Neurol Neurosurg Psychiatry. 2013 Jun; PMID: 23250964

Ricotti V, Ridout D, Scott E, Mayhew A, Main M, Manzur AY, Muntoni F, on behalf of NorthStar Clinical Network .
The Northstar ambulatory assessment in Duchenne muscular dystrophy: considerations for the design of clinical trials. J Neurol Neurosurg Psychiatry. 2015 Mar 2. PMID: 25733532

Ricotti V, Jägle H, Theodorou M, Moore TA, Muntoni F, Thompson DA.
Ocular and neurobehavioural features of Duchenne muscular dystrophy: a signature dystrophin function in the central nervous system. Eur J Hum Genet. 2015 Jun 17. [Epub ahead of print] PMID: 26081639

Ricotti V, Mandy WP, Scoto M, Entwistle K, Messina S, Deconinck N, Pane M, La Foresta S, Lien E, Bajot S, Vita GL, Mercuri E, Skuse DH, Muntoni F.
The wide spectrum of neurodevelopmental disorders in DMD in relation to underlying dystrophin gene mutations. (Dev Med Child Neurol. 2015 Sep 14. [Epub ahead of print] PMID: 26365034)

Ricotti V, Evans MRB, Sinclair CDJ, Butler JW, Ridout DA, Hogrel JY, Emira A, Morrow JM, Reilly MM, Hanna MG, Janiczek RL, Matthews PM, Yousry TA, Muntoni F and Thornton JS.
Upper limb evaluation in Duchenne muscular dystrophy: fat-water quantification by MRI muscle force and function define endpoints for clinical trials (Under review)

Ricotti V, Muntoni F, Voit T. Challenges of clinical trial design for Duchenne muscular dystrophy. 2015 Oct 23. PMID: 26584589

Publications related to the work of this thesis:

Ricotti V, Roberts RG, Muntoni F.
Dystrophin and the brain. Dev Med Child Neurol. 2011 Jan;53(1):12. PMID: 21

Ricotti V, Ridout DA, Muntoni F.
Steroids in Duchenne muscular dystrophy. Neuromuscul Disord. 2013 Aug 2013. PMID: 23856079

Anthony A, Arechavala-Gomeza V, **Ricotti V**, Torelli S, Feng L, Tasca T, Guglieri M, Barresi R, Armaroli A, Ferlini A, Bushby K, Straub V, Ricci E, Sewry C, Morgan J, Muntoni F.
Biochemical characterisation of Becker Muscular Dystrophy patients with DMD deletions mimicking exon 44 and 45 skipping. JAMA Neurol. 2013 Nov 11. PMID: 24217213

De Sanctis R; Pane M; Sivo S; **Ricotti V**; Baranello G; Frosini S; Mazzone E; Bianco F; Fanelli L; Main M; Corlatti A, D'Amico A; Colia G; Scalise R; Palermo C; Alfonsi C; Tritto G; Romeo DM; Graziano A; Battini R; Morandi L; Bertini E; Muntoni F, Mercuri E.
Suitability of North Star Ambulatory Assessment in young boys with Duchenne Muscular dystrophy. Neuromuscul Disord. 2015 Jan;25(1):14-8. PMID: 25454732

Voit T, Topaloglu H, Straub V, Muntoni F, Deconinck N, Campion G, De Kimpe SJ, Eagle M, Guglieri M, Hood S, Liefaard L, Lourbakos A, Morgan A, Nakielny J, Quarcoo N, **Ricotti V**, Rolfe K, Servais L, Wardell C, Wilson R, Wright P, Kraus JE.

Safety and efficacy of drisapersen for the treatment of Duchenne muscular dystrophy (DEMAND II): an exploratory, randomised, placebo-controlled phase 2 study. Lancet Neurol. 2014 Oct. PMID: 25209738

Florence J, Main M, Bianco F, Henrikson E, Servais L, Campion G, Vroom E, **Ricotti V**, Goemans N, McDonald C, Mercuri E; Performance of the Upper Limb Working Group. Dev Med Child Neurol. Development of the Performance of the Upper Limb module for Duchenne muscular dystrophy. Mayhew A, Mazzone ES, Eagle M, Duong T, Ash M, Decostre V, Vandenhauwe M, Klingels K, 2013 Nov. PMID: 23902233.

Panea M, Scalisea R, Berardinelli A, D'Angelo G, **Ricotti V**, Alfierie P, Moroni I, Hartley L, Carmela Pera M, Baranello G, Caterucciae M, Casalino T, Romeo D, Graziano A, Gandioli C, Bianco F, Lombardo ME, Scoto MC, PalermoC, Gualandi F, Ferlini A, Bertini E, Muntoni F, Mercuri E.
Early neurodevelopmental assessment in Duchenne muscular dystrophy. Neuromuscul Disord. 2013. PMID: 23535446

Pane M, Messina S, Bruno C, D'Amico A, Villanova M, Brancaleone B, Bianco F, Striano P, Battaglia D, Lettori D, Vita GL, Bertini E, **Ricotti V**, Mercuri E.
Duchenne muscular dystrophy and epilepsy: phenotype genotype correlations. Neuromuscul Disord. 2013 Apr. PMID: 23465656

Cazzella V, Martone J, Pinnarò C, Santini T, Twayana Shyam S, Sthandier O, D'Amico A, **Ricotti V**, Bertini E, Muntoni F and Bozzoni I .
Exon 45 skipping through U1-snRNA antisense molecules recovers the Dys-nNOS pathway and muscle differentiation in human DMD myoblasts. Mol Ther. 2012 Nov;20(11):2134-42. PMID: 22968481

Mercuri E, McDonald C, Mayhew A, Florence J, Mazzone E, Bianco F, Decostre V, Servais L, **Ricotti V**, Goemans N, Vroom E.
International workshop on assessment of upper limb function in Duchenne Muscular Dystrophy: Rome, 15-16 February 2012. Neuromuscul Disord. 2012 Nov;22(11):1025-8. PMID: 22795657

Mazzone ES, Vasco G, Palermo C, Bianco F, Galluccio C, **Ricotti V**, Castronovo AD, Mauro MS, Pane M, Mayhew A, Mercuri E.
A critical review of functional assessment tools for upper limbs in Duchenne muscular dystrophy. Dev Med Child Neurol. 2012 Jun PMID: 22713125

Pane M, Lombardo ME, Alfieri P, D'Amico A, Bianco F, Vasco G, Piccini G, Mallardi M, **Ricotti V**, Ferlini F, Gualandi F, Vicari S, Bertini E, Berardinelli A, Mercuri E.
ADHD and cognitive function in Duchenne muscular dystrophy: phenotype-genotype correlation . J Pediatr. 2012 Oct;161(4):705-709. PMID: 22560791

Tinsley J; **Ricotti V**; Spinty S; Roper H; Hughes I; Tejura B; Robinson N; Layton G; Davies K; Muntoni F.
Safety, Tolerability and Pharmacokinetics of SMT C1100, a 2-arylbenzoxazole Utrophin Modulator, Following Single- and Multiple-Dose Administration to Pediatric Patients with Duchenne Muscular Dystrophy. (Under review)

UK NorthStar Clinical Network

Prof F Muntoni, Dr A Manzur, Dr S Robb, Dr R Quinlivan, Dr A Sarkozy, Dr V Ricotti, M Main, J Butler, Dubowitz Neuromuscular Centre, Great Ormond Street Hospital for Children NHS Trust, London

Prof K Bushby, Prof V Straub, Dr M Guglieri, Dr M Eagle, Dr A Mayhew, Institute of Human Genetics, Newcastle

Dr H Roper, H McMurchie, Birmingham Heartlands Hospital

Dr A Childs, Dr K Pysden, L Pallant, Yorkshire Regional Muscle Clinic, Leeds General Infirmary

Dr S Spinty, Dr G Peachey, A Shillington, Alder Hey Hospital, Royal Liverpool Children's NHS Trust, Liverpool

Dr E Wraige, Dr H Jungbluth, J Sheehan, R Spahr, Evelina Children's Hospital, St Thomas' Hospital, London

Dr I Hughes, E Bateman, C Cammiss, Royal Manchester Children's Hospital, Manchester

Dr T Willis, L Groves, N Emery, The Muscle Clinic, Robert Jones & Agnes Hunt, Orthopaedic & District Hospital NHS Trust, Oswestry

Dr P Baxter, M Senior, E Scott, Sheffield Children's Hospital NHS Trust, Sheffield

Dr L Hartley, B Parsons, University Hospital of Wales, Cardiff

Dr A Majumdar, L Jenkins, B Toms, Frenchay Hospital, Bristol

Dr K Naismith, A Keddie, Armistead Child Development Centre, Kings Cross Hospital, Dundee

Dr I Horrocks, M Di Marco, Royal Hospital for Sick Children, Yorkhill, Glasgow

Dr G Chow, A Miah, Queens Medical Centre, University Hospital, Nottingham

Dr C de Goede, Preston Royal Hospital

Dr N Thomas, M Geary, J Palmer, Southampton General Hospital

Dr C White, K Greenfield, Morriston Hospital, Swansea

I Wilson, NorthStar coordinator, Dubowitz Neuromuscular Centre, Great Ormond Street Hospital for Children NHS Trust, London

Italian Neuromuscular Network

Dr S. Messina Department of Neurosciences, University of Messina and Centro Clinico Nemo Sud, Messina

Dr A. Berardinelli Child Neurology and Psychiatry Unit, "C. Mondino" Foundation, Pavia

Dr G. Comi Dino Ferrari Centre, Neuroscience Section, Department of Pathophysiology and Transplantation (DEPT), University of Milan, Neurology Unit, Ca' Granda Ospedale Maggiore Policlinico, Milan

Dr A. D'Amico, Dr E. Bertini, Department of neurosciences, unit of neuromuscular and neurodegenerative disorders, Bambino Gesù Children's Hospital, Rome

Dr C. Bruno Center of Myology and Neurodegenerative Disease", Istituto Giannina Gaslini, Genova

Dr L. Politano, Cardiomyology and Medical Genetics, Department of Experimental Medicine, Second University of Naples

Dr R. Battini, Department of Developmental Neuroscience, Stella Maris Institute, Pisa

E. Pegoraro Department of Neurosciences, University of Padua, Padua

A. Pini Child Neurology and Psychiatry Unit, IRCCS Istituto delle Scienze Neurologiche di Bologna

T. Mongini Department of Neurosciences 'Rita Levi Montalcini', University of Torino

L. Morandi Developmental Neurology Unit, Istituto Neurologico "Besta" Milan